



Exploration of natural compounds as sources of new bifunctional scaffolds targeting cholinesterases and beta amyloid aggregation: The case of chelerythrine

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ABSTRACT

The presented project started by screening a library consisting of natural and natural based compounds for their acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity. Active compounds were chemically clustered into groups and further tested on the human cholinesterases isoforms. The aim of the presented study was to identify compounds that could be used as leads to target two key mechanisms associated with the AD's pathogenesis simultaneously: cholinergic depletion and beta amyloid (A β) aggregation. Berberin, palmatine and chelerythrine, chemically clustered in the so-called isoquinoline group, showed promising cholinesterase inhibitory activity and were therefore further investigated. Moreover, the compounds demonstrated moderate to good inhibition of A β aggregation as well as the ability to disaggregate already preformed A β aggregates in an experimental set-up using HFIP as promotor of A β aggregates. Analysis of the kinetic mechanism of the AChE inhibition revealed chelerythrine as a mixed inhibitor. Using molecular docking studies, it was further proven that chelerythrine binds on both the catalytic site and the peripheral anionic site (PAS) of the AChE. In view of this, we went on to investigate its effect on inhibiting A β aggregation stimulated by AChE. Chelerythrine showed inhibition of fibril formation in the same range as propidium iodide. This approach enabled for the first time to identify a cholinesterase inhibitor of natural origin—chelerythrine—acting on AChE and BChE with a dual ability to inhibit A β aggregation as well as to disaggregate preformed A β aggregates. This compound could be an excellent starting point paving the way to develop more successful anti-AD drugs.

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1. Introduction

Alzheimer's disease (AD) is a seriously damaging neurodegenerative disease affecting human health. The most important changes observed in the brain of AD patients are the appearance of amyloid beta (A β) plaques, neurofibrillary tangles and a dramatic decrease in hippocampal and cortical levels of the neurotransmitter acetylcholine (ACh). A wealth of evidence indicates that the pathological accumulation of A β in the brain relates to a variety of damaging events that ultimately causes neuronal destruction and cholinergic deficit.¹

Impairment of ACh-mediated neurotransmission involving ACh depletion is a key component of AD, thus the rationale behind the use of cholinesterase inhibitors (ChEI) in this pathology has primarily consisted in providing an amplification of the deprived cholinergic

activity. The ChEI donepezil, galanthamine and rivastigmine represent the first line pharmacotherapy for mild to moderate AD. Interest in ChEI has also increased due to findings supporting cholinesterase's involvement in β -amyloid peptide fibril formation during AD pathogenesis.² Various studies have indeed supported that ChEI can prevent A β oligomerization, thus displaying both anti-amyloid and neuroprotective disease-modifying effects.³

The peripheral anionic site (PAS) of AChE was identified as the locus where the interaction between the enzyme and A β apparently takes place.⁴ By contrast, no significantly enhanced A β _{1–40} fibril formation was observed when co-incubating the peptide with BChE.² In fact, after several years of research with cholinesterases, the connection of BChE with amyloidogenesis still remains unclear.^{5,6} However, it has been established that in the status of advanced AD, BChE hydrolyses the already depleted acetylcholine levels thus exacerbating the cholinergic imbalance.⁷ Moreover, Holmes et al. proposed that low-activity BChE in AD patients correlates with better cognitive function.⁸ In view of that, our study did not only

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concentrate on AChE inhibition, but also included BChE. We therefore focused on the identification of dual inhibitors of AChE and BChE, which could increase the likelihood of finding promising leads/drug candidates against AD. Based on the complex multifactorial nature of AD, developing compounds able to act on multiple targets within the biological network simultaneously and thus retrieve the biological equilibrium has been claimed as a successful approach.⁹ This strategy can be achieved in several ways. The combined administration of AChE inhibitors together with NMDA receptor antagonists is one example of this approach. However, it has the major drawback that every compound has to be tested by its own as well as in combination in terms of safety and efficiency. Another way is to identify/develop a single drug able to act as multi-target-directed ligand. Some examples are the synthetic AChE inhibitors ladostigil (TV-3326) and memoquin. Ladostigil, currently being evaluated in clinical studies, also inhibits the monoamine oxidase, modulates the amyloid precursor protein processing and has antioxidant properties.¹⁰ In turn, memoquin exhibits antioxidant functions, acts as β -secretase inhibitor, A β anti-aggregant and influences tau hyperphosphorylation.¹¹ In particular, molecules with ability to destabilize preformed A β aggregates together with inhibitory properties of A β aggregation and cholinesterases activity could be regarded as especially desirable.

So far, two of the three ChEIs licensed for the treatment of dementia in western countries are of natural origin (galanthamine and rivastigmine, a semi-synthetic derivative of physostigmine) and another natural compound, huperzine A, isolated from *Huperzia serrata* (Thunb.), is a licensed cholinesterase inhibitor used as anti-AD drug in China.¹² Therefore, it seems likely to find new anti-AD pharmacophores when exploring natural sources. Moreover, natural-based compounds are often better tolerated than their synthetic congeners which is a favorable aspect on the process of approval of new drugs.¹³ Thus, in this contribution we screened a commercial library of natural and natural-based compounds (Supplementary Table S1) for their AChE and BChE inhibitory activity. Of the whole library (502 compounds) 23 compounds were identified as either active on AChE, BChE or both (dual inhibitors), and they were clustered into six different groups according to their chemical structures and not according to their biosynthetic routes, which is the general way of classifying natural compounds. Follow-up studies of the compounds were conducted and the identification of structural key requirements necessary for the biological activity was attempted.

2. Results and discussion

2.1. Bioactivity screening

The screening of the natural compound library for inhibition of AChE's and BChE's hydrolase activity was done using a kinetic assay based on Ellman's reaction.¹⁴ To ensure high quality from the screening viewpoint, the following statistical parameters were used to evaluate the assay: signal-to-noise (S/N) ratio, signal-to-background (S/B) ratio, signal window coefficient (Z' factor) and coefficient of variation of the assay (CV_A). An assay with $Z' \geq 0.5$ is regarded as a good-to-excellent assay, depending on the proximity to $Z' = 1$ (the ideal assay).¹⁵ In the screening presented herein the acceptance parameters were set as: $Z' > 0.5$, $S/N > 15$ and $S/B > 15$. Three times the standard deviation of the maximal signal (negative controls, samples containing the uninhibited enzyme) was used to determine the theoretical hit limit, which resulted in 40% of inhibition. However, since the screening was planned at an average compound concentration in the micromolar range (17.5 μ M), the threshold was empirically set higher, at 50% inhibition, to identify more potent inhibitors.

In total, 23 compounds displayed inhibitory activity towards AChE (electric eel origin) and/or BChE (horse origin). The primary screening results of all the tested compounds can be found in the Supplementary Table S1. Both non-human enzymes are frequently used in primary screening campaigns because they represent a cost-effective way to generate a first sound bioactivity profile of compound libraries.¹⁶ Compounds were regarded as active if they scored over 50% inhibition of the hydrolase activity. This activity limit guaranteed to identify compounds with IC_{50} values below 33 μ M, the highest compound concentration in the primary screening assay.

Figure 1 represents the results of the initial screening against AChE and BChE. Three compounds were able to inhibit more than 50% of the activity of both cholinesterases and are located in quadrant I (represented by triangles). Quadrant II includes six compounds (circles) showing an AChE inhibition over 50% but with a BChE inhibition lower than 50% thus giving initial evidence that these compounds can be regarded as selective AChE inhibitors. Conversely, the 14 compounds located in the quadrant IV (squares), were found as selective BChE inhibitors. The majority of the compounds in the library, located in quadrant III (diamonds), did not exhibit more than 50% inhibition neither on AChE nor on BChE.

2.2. Exploration of the chemical space occupied by the library

After screening the collection on AChE and BChE inhibition, the substances were mapped using ChemGPS-NP. Based on a principle component analysis (PCA) with eight dimensions describing 2D physico-chemical parameters (e.g., size, shape, aromaticity, lipophilicity and flexibility) for a reference set, ChemGPS-NP allows to map new compounds into the chemical space.¹⁷ Supplementary Figure S1 shows that the compounds are widely distributed over the chemical space. However, the 23 active hits inhibiting ChEs (selective for AChE or BChE as well as unselective inhibitors) clearly locate within the same area (colored spheres). Thus, it can be concluded, that compounds with limited flexibility (PC4) and low molecular weight (PC1), a certain degree of aromaticity (positive PC2) as well as lipophilicity (positive PC3) possess a higher likelihood to display ChE inhibition. This information can be compared with data we have obtained from a synthetic library consisting of compounds based on naturally existing scaffolds that was screened toward selective BChE inhibition.^{18,19} Active representatives of this library were located in the same region of the chemical space as the compounds identified as ChE inhibitors in the present study. Only the flexibility (as determined by PC4) differed:

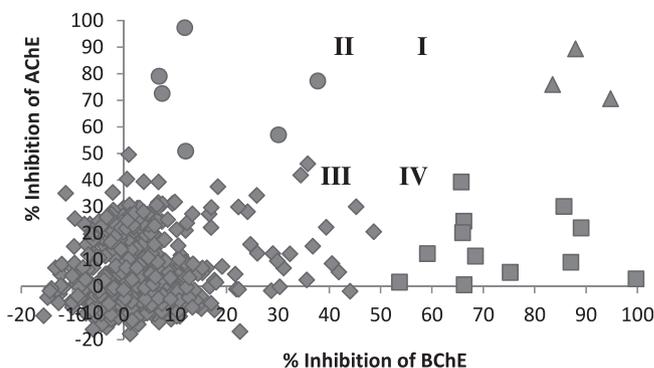


Figure 1. Correlation plot of the electric eel AChE and horse BChE inhibitory activities of the entire natural compound library (502 compounds). Unselective ChE inhibitors are located in quadrant I (triangles), quadrant II shows selective AChE inhibitors (circles), quadrant III contains all inactive compounds (diamonds) while the selective BChE inhibitors are found in quadrant IV (squares; only 12 data points are visible in the graph instead of 14 due to an overlap among the compounds).

synthetic diarylimidazoles seemed to require a higher degree of flexibility to act as BChE inhibitors when compared to natural products. However, molecular flexibility is a property that seems to differ between natural and synthetic molecules, with natural compounds typically displaying less flexibility than their synthetic counterparts.²⁰ This is also reflected in the compound library used herein as only approximately a quarter of the natural compounds were located on the positive side of the PC4 axis. Elucidation of the chemical space occupied by the active hits thus revealed valuable information about the physico-chemical parameters that are essential for ChE inhibition.

2.3. Chemical clustering of the active compound groups

To continue with an in-depth characterization of the active molecules, an exclusion criterion was applied to the general group of 23 hits. The criterion was based on: (i) if they were known ChE inhibitors and (ii) if they have poor chemical stabilities. Following the first element, the known ChE inhibitors galanthamine, ebelactone B, huperzine A and physostigmine were excluded from further investigation. However, their identification demonstrated once more the accuracy of the kinetic assay used. Following the second element, the original hits solasodine, tetrahydroalstonine and harmine were also excluded, as they were shown to be chemically unstable in the assay buffer during the reconfirmation process (data not shown). Thus, a total of 16 hits out of the original 23 were taken up for further investigation (Tables 1 and 2). The results presented earlier (Fig. 2) suggested that the active hits shared a specific chemical space, most likely given by common chemical scaffolds. Indeed, upon close examination of these molecules, the remaining 16 compounds were found to share common scaffolds and based on those similarities they were structurally clustered into six groups (Tables 1 and 2). This structural sorting provided a better view towards the most promising and interesting functional moieties within the natural compounds studied.

2.3.1. Quinazolines

The first group (Table 1) comprises compounds with the quinazoline scaffold and includes peganole, vasicine and desoxypeganine. The first two (peganole and vasicine) were shown to be selective BChE inhibitors and hence were further studied concerning their activity towards the human BChE (hBChE). Peganole was less active compared to the horse BChE with an IC_{50} value higher than $20 \mu\text{M}$ but vasicine showed good inhibitory activity also against the hBChE ($3.13 \pm 0.79 \mu\text{M}$). Desoxypeganine displayed similar activity on both BChE and AChE indicating that it was a non-selective inhibitor. From a chemical point of view it is highly interesting that these three representatives differ in the position and/or presence of only one hydroxy group. In peganole and vasicine, the position of the OH group determines the potential of the BChE inhibition and indicates that OH-substitution in position 9 (as in the vasicine) favors the selective enzyme inhibition whereas an OH group in position 4 of the quinazoline scaffold (present in peganole) results in a decrease of activity. The loss of the hydroxy group as is the case in desoxypeganine results in an unselective inhibition of both enzymes.

The quinazoline scaffold has been used as a model for the synthesis of hybrid compounds out of quinazolinimines and lipoic acid with the most active compound showing an IC_{50} value on BChE of 5.7 nM (82-fold higher than the potency toward AChE).²¹ In another study, the covalent connection of two quinazolinimine molecules by different hydrocarbon spacers resulted in one highly potent and selective BChE inhibitor (IC_{50} of 3 nM).¹⁶ Moreover, a quinazoline derivative, coded as SMER28, has been described as an enhancer of autophagy, a cellular pathway responsible for the degradation of aggregated proteins, that is, beta amyloid

aggregates.²² Thus, because this quinazoline chemical class has already attracted the attention of medicinal chemists, we did not further investigate it.

2.3.2. Indoles

The second group (Table 1) consists of four indole representatives which were found to be selectively active on BChE, with IC_{50} values ranging from 4.97 to $10.63 \mu\text{M}$. Interestingly, three out of the four compounds (nitrarine, hirsutine and catharanthine) showed slightly higher activity towards the human BChE when compared to the horse enzyme. As can be seen from these data, the side chain fused to the indole motif does not critically influence the potential of the BChE inhibition. Indole derivatives have not been intensively investigated in terms of their ability to inhibit cholinesterases in the context of AD. Recently, Pereira et al. studied the inhibitory effect of *Catharanthus roseus* root alkaloids on AChE and described catharanthine as not active.²³ This is in accordance with our results as we identified this indole derivative as a selective BChE inhibitor, not active upon AChE. To the best of our knowledge, no biological data have been described so far for nitrarine. Rauwolscine, also known as isoyohimbine or alpha-yohimbine, is a known highly potent α_2 -adrenergic receptor antagonist.²⁴ In 1999, Shimada et al. reported a protective effect of hirsutine on glutamate-induced neuronal death.²⁵ Moreover, it also showed a vasodilating activity in the same micromolar range²⁶ as the herein described selective BChE activity. Due to the fact that representatives of this group exhibited only modest BChE inhibitory activity we decided not to continue with more follow-up studies.

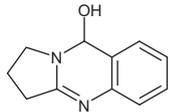
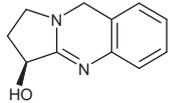
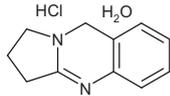
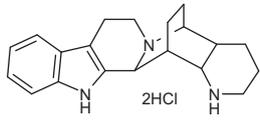
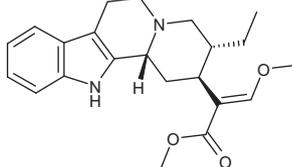
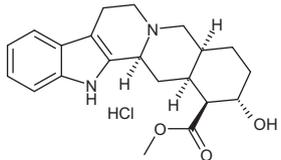
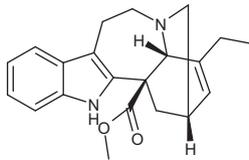
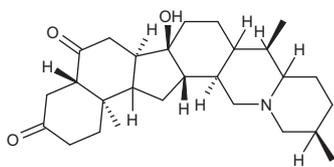
2.3.3. Steroid alkaloids

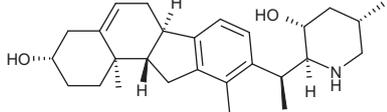
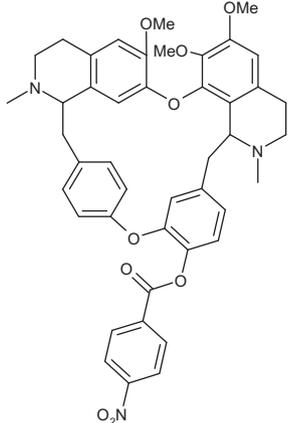
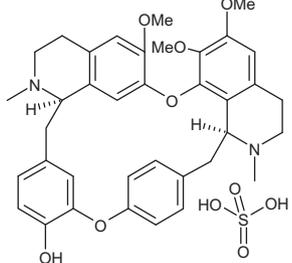
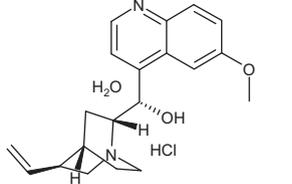
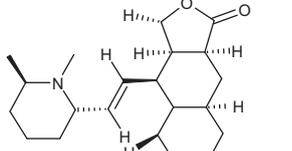
Within the group of active steroid alkaloids (Table 1), sevedindione and veratramine caused a selective inhibitory activity towards BChE. For sevedindione, no biological activities have been described so far. This compound showed an IC_{50} value on horse BChE of $3.68 \pm 0.26 \mu\text{M}$ and displayed an approximately ten times higher potency on human BChE with an IC_{50} of $0.27 \pm 0.04 \mu\text{M}$. Veratramine showed a weaker activity than sevedindione towards horse BChE (IC_{50} of 10.78 ± 1.13) and human BChE (IC_{50} of $19.46 \pm 0.95 \mu\text{M}$). Wang et al. proved a hypotensive effect of veratramine in in vivo experiments and also demonstrated a decrease in heart rate under the influence of this compound.²⁷ Moreover, they did not observe any side effects during their studies. Compared to veratramine, sevedindione possesses a continuous steroidal ring system together with a tertiary nitrogen. Sevedindione has good inhibitory potential of the human enzyme, but lower if compared to various compounds (i.e., phenothiazines, cymserine analogues and isosorbide-based compounds) that have been recently reported as selective BChE inhibitors.^{28–30} Therefore, this group was also excluded from further investigations.

2.3.4. Polycyclic ring systems

E6 berbamine and oxyacanthine sulfate are clustered together within the group of polycyclic ring systems. Both compounds displayed similar activity on horse BChE with $4.20 \pm 0.21 \mu\text{M}$ for E6 berbamine and $4.10 \pm 0.20 \mu\text{M}$ for oxyacanthine sulfate. Moreover, both compounds showed a two-fold improved activity on the human enzyme (E6 berbamine $2.14 \pm 0.07 \mu\text{M}$ and oxyacanthine sulfate $1.89 \pm 0.07 \mu\text{M}$). These compounds mainly vary in the substitution pattern of the (substituted) hydroxy group as well as the position of the ether bridge connecting the two phenyl rings. E6 berbamine bears a *para*-nitro-benzoyl substituent on the OH group, as opposed to the oxyacanthine sulfate which has a free hydroxyl group. As no differences in activity were detected between these two molecules, it seems that the electronic and steric effects of the *para*-nitro-benzoyl substituent are not relevant.

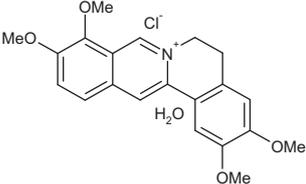
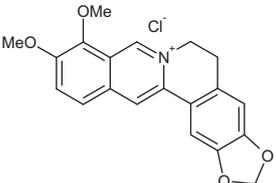
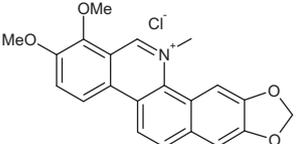
Table 1
Clustered chemical groups from the compounds present in the natural products collection and their natural sources

Group	Compound	Examples of plant sources	Structure	IC ₅₀ (μM)		
				BChE	hBChE	AChE
Quinazoline	Peganole	<i>Peganum harmala</i> L. ⁴⁹		11.39 ± 0.37	>20	n.a.
	Vasicine	<i>Peganum harmala</i> L. ⁴⁹		2.53 ± 0.36	3.13 ± 0.79	n.a.
	Desoxypeganine	<i>Peganum harmala</i> L. ⁴⁹		11.94 ± 0.05	n.t.	11.81 ± 0.06
Indole	Nitrarine	<i>Nitraria schoberi</i> L. ⁵⁰		10.63 ± 0.37	9.01 ± 0.22	n.a.
	Hirsutine	<i>Uncaria</i> spp. ⁵¹		4.97 ± 0.33	1.97 ± 0.04	n.a.
	Rauwolscine	<i>Rauwolfia</i> spp. ⁵²		8.38 ± 0.54	13.68 ± 1.48	n.a.
	Catharanthine	<i>Vinca rosea</i> L. ⁵³		5.17 ± 0.18	3.17 ± 0.11	n.a.
Steroid alkaloid	Sevedindione	Synthetic derivative of sevedine (<i>Korolkowia sewerzowii</i> Regel) ⁵⁴		3.68 ± 0.26	0.27 ± 0.04	n.a.

	Veratramine	<i>Veratrum album, Veratrum viride</i> ⁵³		10.78 ± 1.13	19.46 ± 0.95	n.a.
Polycyclic ring system	E6 Berbamine	Synthetic derivative of berbamine (<i>Berberis amurensis</i> Rupr.) ⁵⁵		4.20 ± 0.21	2.14 ± 0.07	n.a.
	Oxyacanthine sulfate	<i>Berberis aquifolium</i> Pursh ⁵⁶		4.10 ± 0.20	1.89 ± 0.07	n.a.
Mixed chemical entity	Quinidine	<i>Cinchona</i> spp. ⁵³		7.37 ± 0.03	1.23 ± 0.07	n.a.
	Himbacine	<i>Galbulimima baccata</i> F.H.Bailey ⁵⁷		n.a.	n.a.	22.72 ± 0.64

Potencies (IC₅₀ values in μM) obtained during the follow-up investigation of the hits in either cholinesterase are presented. BChE = horse BChE; hBChE = human BChE; AChE = electric eel AChE; n.a. = not active; n.t. = not tested.

Table 2
Overview of the biological activity (IC₅₀ values in μM) of the isoquinoline group together with their natural sources

Group	Compound Structure	Examples of plant sources	IC ₅₀ (μM)						
			AChE	hAChE	BChE	hBChE	A β aggreg	A β disaggreg	
	Palmatine								
Isoquinoline		<i>Phellodendron amurense</i> Ruprecht, <i>P. chinense</i> Schneid ⁵⁸	4.07 \pm 0.09	1.69 \pm 0.11	n.a.	n.t.	92.15 \pm 3.42	174.30 \pm 10.30	
	Berberine								
		<i>Berberis</i> , <i>Hydrastis</i> , <i>Coptis</i> , <i>Thalictrum</i> spp. ⁵³	2.74 \pm 0.22	0.61 \pm 0.04	n.a.	n.t.	43.84 \pm 6.09	105.90 \pm 5.90	
	Chelerythrine								
		<i>Chelidonium majus</i> L., <i>Sanguinaria canadensis</i> L., <i>Dicranostigma lactuoides</i> Hook. f. et. Thoms., <i>Macleaya</i> , <i>Bocconia</i> , <i>Zanthoxylum</i> spp. ⁵⁹	3.78 \pm 0.15	1.54 \pm 0.07	6.33 \pm 0.93	10.34 \pm 0.24	4.20 \pm 0.43	13.03 \pm 2.89	

AChE = electric eel AChE; hAChE = human AChE; BChE = horse BChE; hBChE = human BChE; A β _{1–40} aggreg. = inhibition of A β _{1–40} aggregation; A β _{1–40} disaggreg. = disaggregation of preformed A β _{1–40} aggregates; n.a. = not active; n.t. = not tested.

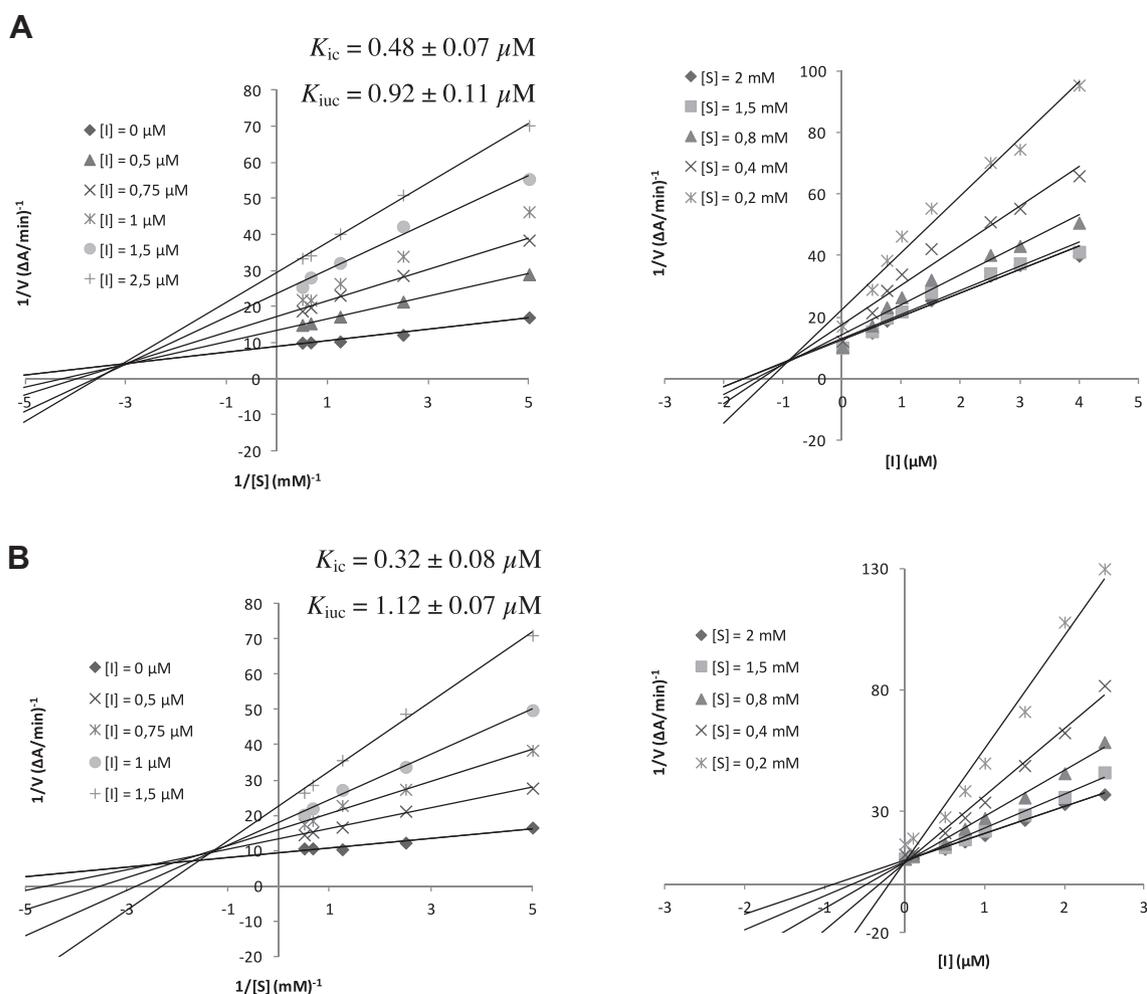


Figure 2. Lineweaver–Burk (left panel) and Dixon (right panel) plots with different substrate concentration [S] in the presence of varying chelerythrine concentrations [I] on electric eel AChE (A) and human AChE (B).

So far, there are no reports present describing their impact on the ChE activity or beneficial effects for the treatment of AD. Because of a lack of AChE inhibitory activity of both representatives, it was decided not to perform any follow-up studies with this compound group.

2.3.5. Mixed chemical entities

The compounds quinidine and himbacine are the active representatives of this group. They differ completely from each other and from the rest of the compound clusters in terms of their chemical structures. Quinidine possesses a cinchonan scaffold whereas himbacine represents a decahydronaphthofuranone derivative. The well-known antiarrhythmic agent quinidine was proven to be a selective BChE inhibitor with good activity towards the horse enzyme ($7.37 \pm 0.03 \mu\text{M}$) and even higher potency in inhibiting the human form, with an IC_{50} value of $1.23 \pm 0.07 \mu\text{M}$. The second compound within this group, himbacine, showed no activity towards BChE in the primary screening (30.1% inhibition at $10 \mu\text{M}$) but was selected as active concerning its AChE inhibitory activity. Thus, the IC_{50} value on AChE was determined and found to be $22.7 \pm 0.6 \mu\text{M}$. Himbacine is known as a potent antagonist of the M(1) subtype of the muscarinic receptor³¹ which might be a disadvantage in the treatment of Alzheimer's disease since muscarinic receptor agonists have shown to cause improvement of cognitive function and reversal of cognitive deficits.³² This fact together with the low potency of himbacine against the AChE (lowest among all

the hits) prompted us not to further investigate it on the human AChE.

2.3.6. Isoquinolines

Palmatine, berberine as well as the benzophenanthridine alkaloid chelerythrine are clustered together in the isoquinolines group (Table 2). All three compounds showed good inhibitory activity towards electric eel AChE. Berberine and palmatine displayed a selective inhibition of the hydrolytic activity of AChE and showed IC_{50} values of 2.74 ± 0.22 and $4.07 \pm 0.09 \mu\text{M}$, respectively. Chelerythrine also showed a moderate activity towards AChE ($3.78 \pm 0.15 \mu\text{M}$). Moreover, this compound further exhibited activity on horse BChE with an IC_{50} value of $6.33 \pm 0.93 \mu\text{M}$. The three compounds' activities towards the human enzymes were also evaluated. Both berberine and palmatine showed increased activity towards the human AChE, berberine being the most active one ($0.61 \pm 0.04 \mu\text{M}$). Interestingly, chelerythrine also showed slightly higher activity towards the human AChE ($1.54 \pm 0.07 \mu\text{M}$, Table 2) than towards the electric eel AChE ($3.78 \pm 0.15 \mu\text{M}$). A reversed situation was seen against BChE, as chelerythrine was slightly more active towards the horse BChE ($6.33 \pm 0.95 \mu\text{M}$) when compared to the human enzyme ($10.34 \pm 0.24 \mu\text{M}$, Table 2). The only difference in the chemical structure of palmatine and berberine is the substitution pattern at the dihydroisoquinoline structure. According to these data, a dioxymethylene substitution is preferred when compared to vicinal dimethoxy substituents. Moreover, the

increased aromaticity and lipophilicity of chelerythrine due to its phenanthridine backbone seem to favor the inhibition of both cholinesterases.

Compared to the compound groups presented earlier, chelerythrine offers the most attractive compound with good inhibitory potential of both the AChE and the BChE. Also, from a medicinal chemistry perspective, the basic scaffold of the isoquinoline group could be regarded as a good starting point for chemical elaborations. Therefore, we selected them, in particular the dual ChE inhibitor chelerythrine, to proceed with follow-up studies.

2.4. Kinetic mechanism of AChE inhibition

Kinetic studies were performed to determine the inhibition mechanisms of chelerythrine on the electric eel and the human AChE. The mechanism of inhibition was graphically determined by applying the Lineweaver–Burk and Dixon plots. The kinetic curves revealed that chelerythrine is a mixed inhibitor of electric eel AChE with a slightly higher competitive behavior ($K_{ic} = 0.48 \pm 0.07 \mu\text{M}$ and $K_{iuc} = 0.92 \pm 0.11 \mu\text{M}$, Fig. 2A). The same mechanism of inhibition was found using the human AChE with $K_{ic} = 0.32 \pm 0.08 \mu\text{M}$ and $K_{iuc} = 1.12 \pm 0.07 \mu\text{M}$ (Fig. 2B). Due to the mixed kinetic mechanism it can be tentatively speculated that chelerythrine can bind to the PAS.^{4,33} Hence, we performed docking studies to elucidate possible interactions between chelerythrine and the AChE.

2.5. Docking results of chelerythrine on AChE

The crystal structure of 1FSS (from *Torpedo californica*) was used as a model receptor for docking chelerythrine, as this structure is fundamentally similar to the human AChE and it has been extensively used in research. The active site of the AChE consists mainly of residues TYR70, TRP84, GLY118, GLY119, GLU200, SER201, ALA226, TRP279, TYR327, PHE330 and HIS440, respectively. Chelerythrine covers the active gorge site showing a hydrogen bond interaction with TYR130 as well as π -stacking interactions with TYR121 and TYR334 PAS residues (Fig. 3), indicating that this compound can be a very interesting inhibitor with an additional capacity to target the A β aggregation process. Water molecules are shown to play an important role in binding of the inhibitors within the active site of the enzyme. In this case, conserved water

molecules from 1FSS (710, 718, 722, 728) denoted as W1, W2, W3 and W4 as shown in Figure 3 are found within the catalytic site and PAS regions, respectively, and may mediate hydrogen bonding interactions with the active site residues.

2.6. Inhibition of A β aggregation

In view of earlier results and given the key contribution of amyloid β assembly to AD pathogenesis, we focused also on the influence of chelerythrine on amyloid beta 1–40 (A β) aggregate formation. The assay used was based on the typical measurement of the fluorescence changes of thioflavin T (ThT). The fact that the in vitro generation of fluorescent ThT-positive A β aggregates can take several days prompted us to apply an accelerated method using 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as an aggregation formation enhancer.³⁴ Fluorescence changes of the ThT, typical in the presence of aggregates, were obtained by using a minimal amount of A β peptide_{1–40} (0.7 μM) after a relatively short incubation time (45 min) thus allowing a quick identification of active compounds within a reasonable time frame. Chelerythrine displayed an IC_{50} value of $4.20 \pm 0.43 \mu\text{M}$ in inhibiting A $\beta_{1–40}$ aggregation. The reference compounds used here were the nordihydroguaiaretic acid, which displayed a potency of 18 μM , and the 4-aminophenol, which showed an inhibition of A $\beta_{1–40}$ assembly of 64% at 250 μM . In comparison, chelerythrine demonstrated 67% inhibition of A $\beta_{1–40}$ aggregation already at 10 μM . Moreover, synthetic compounds specifically developed to inhibit the A β fibrillogenesis also showed activity in the micromolar range, like for instance 4-hydroxyindole with an IC_{50} value of 85 μM on A $\beta_{1–42}$.³⁵ Thus, this suggests that chelerythrine is a highly active inhibitor of A $\beta_{1–40}$ aggregation.

Berberine and palmatine were also included in these experiments to obtain a better overview of all of the isoquinolines identified herein. Their potency values were located in the upper micromolar range (43.84–92.15 μM) (Table 2). These results are in agreement with recent publications in which the A $\beta_{1–42}$ aggregation inhibitory activities of berberine, benzenediol-hybrid as well as 9-N-substituted berberine have been reported.³³ These authors have found that berberine causes a 36% aggregation inhibition at 20 μM . Their most active derivatives, a hydroquinone-berberine hybrid and a 9-N-substituted berberine compound linked with an *ortho* methyl phenyl ring, showed 92% and 95% inhibition at 20 μM , respectively.

2.7. Induction of A β disaggregation

Having proven the inhibitory properties of these compounds on preventing the A $\beta_{1–40}$ aggregation, we then evaluated if the isoquinolines were able to disaggregate already preformed A $\beta_{1–40}$ aggregates. This assay could be more relevant from a clinical perspective. Disaggregation of existing A β aggregates/fibrils in the AD brain may be indicated especially at the beginning of the treatment to reduce the neurotoxic effects of A β aggregates/fibrils and thus prevent neurodegeneration.

The assay used followed the same general procedure as for studying the inhibition of A $\beta_{1–40}$ aggregation (ThT assay), but to obtain aggregates the protein was allowed to assemble 45 min prior to adding the test compounds. As a positive control for the effect on A $\beta_{1–40}$ disaggregation, resveratrol was used. In this experimental set-up, a potency of $81.80 \pm 3.74 \mu\text{M}$ was registered for resveratrol, which is in accordance to previous results showing that at 10 and 100 μM this compound decreases the ThT fluorescence intensity of A $\beta_{1–42}$ fibrils to 71% and 32%, respectively, after 28 h incubation at 37 °C.³⁶ Chelerythrine, once more, showed a high activity in disaggregating preformed A $\beta_{1–40}$ aggregates with an IC_{50} of $13.03 \pm 2.89 \mu\text{M}$ after 45 min incubation. Few compounds have been found active in A β disaggregation at low

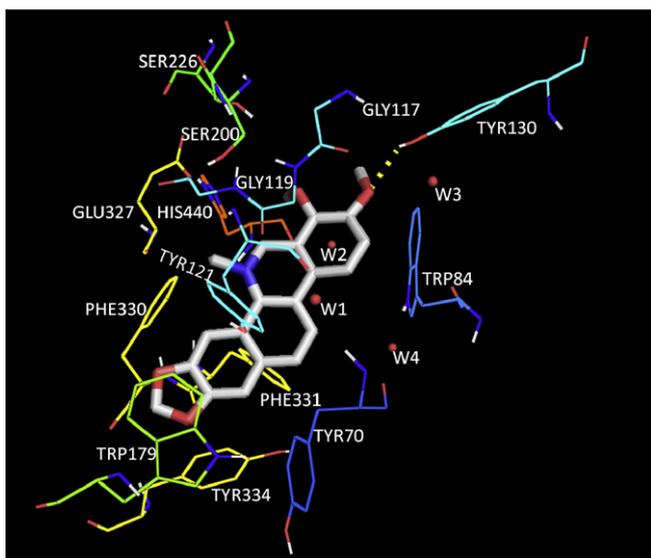


Figure 3. Chelerythrine within the active site of 1FSS (*Torpedo californica* AChE). Water molecules are shown as red spheres.

Table 3
Inhibition of AChE-induced A β_{1-40} fibrillogenesis by chelerythrine and the control compound propidium iodide at different concentrations

Compound	Concentration (μM)	Inhibition of AChE-induced A β fibrillogenesis (%) \pm SD
Chelerythrine	1	0.8 \pm 0.1
	5	48.5 \pm 3.7
	10	65.0 \pm 0.3
	100	88.4 \pm 1.0
Propidium iodide	10	33.9 \pm 4.4
	100	93.1 \pm 2.2

micromolar concentrations. The most potent inhibitors so far are the natural product hyperforin, which reduced A β_{1-40} aggregates to 20% at 100 μM after 4 days incubation, as well as 2,6-diaryl-1,4-benzoquinone derivatives, which displayed IC₅₀ values on A β_{25-35} between 5 and 14 μM also after 4 days incubation.^{37,38} We could further show that berberine and palmatine also demonstrated a remodeling behavior of A β_{1-40} aggregates but with lower activities compared to chelerythrine (IC₅₀ of 105.90 \pm 5.90 and 174.3 \pm 10.3 μM , respectively).

Nordihydroguaiaretic acid, which inhibits the A β aggregate formation, does not influence preformed aggregates (A β_{1-40})³⁹ and resveratrol, which is able to dissolve already preformed A β_{1-42} aggregates, does not show the ability to inhibit the aggregation of A β_{1-42} .³⁶ The already mentioned synthesized 2,5-diaryl and 2,6-diaryl-1,4-benzoquinones displayed a bivalent behavior on A β_{25-35} aggregation³⁷ but have not been proven to act as cholinesterase inhibitors. In the case of chelerythrine, we describe for the first time, to the best of our knowledge, a dual cholinesterase inhibitor acting on AChE and BChE, with additional ability not only to inhibit the A β_{1-40} aggregation but also to disaggregate preformed A β_{1-40} aggregates.

2.8. Inhibition of AChE-induced A β fibril formation by chelerythrine

In contrast to the accelerated method using HFIP as aggregation enhancer, the experimental procedure that uses AChE as promoter of fibril formation resembles better the processes taking place in the AD brain. Inestrosa et al. demonstrated in vitro that recombinant human AChE directly enhances the aggregation of A β_{1-40} peptide into fibrils, forming a stable AChE-A β complex.² The fact that AChE inhibitors are already clinically used to restore the cholinergic balance and that AChE seems to be strongly involved in the A β fibril formation process stresses the importance of identifying compounds that can potentially target the pro-aggregating effect of AChE as well as its hydrolase activity. However, the role of BChE in AD should not be neglected. It is known that BChE is able to compensate for the lower activity of AChE detected at the later stages of the AD pathogenesis⁴⁰ giving good grounds for using compounds which act as dual inhibitors to efficiently slow down the hydrolysis of acetylcholine.

The identification of chelerythrine as mixed inhibitor as well as the results of the docking study, (indicating that this compound likely interacts with PAS), increased the likelihood that the compound may also inhibit AChE-induced A β aggregation. Indeed, as seen in Table 3, chelerythrine inhibited AChE-induced A β aggregation at 5, 10 and 100 μM with 48.5%, 65.0% and 88.4%. No inhibition was detected at a concentration of 1 μM . Propidium iodide was included as a positive control in our experiments at the concentrations 10 and 100 μM , which caused an inhibition of AChE-induced A β fibril formation of 33.9% and 93.1%, respectively. These values are comparable to those found in the literature. An inhibition of 82% at 100 μM of propidium iodide has been reported on

A β_{1-40} .⁴¹ Propidium iodide is a non-competitive AChE inhibitor (IC₅₀ value of 32.2 \pm 2.2 μM on the human AChE) and it has been found to bind to the PAS region of the enzyme.^{2,4} Compounds described in the literature are regarded as good inhibitors of AChE-induced A β fibril formation if they exhibit biological activity in the range of 82–98% at 100 μM .⁴² Compared to these data, chelerythrine can also be classified as a potent inhibitor of AChE-induced A β_{1-40} aggregation. Accordingly, the biological characteristics of chelerythrine described herein make this compound a promising lead for the development of potent AD-modifying drugs.

3. Conclusion

Because of the fact that AD is a complex multifactorial disorder, the use of compounds able to modify several targets simultaneously is considered to be a successful strategy in the treatment of this disease. Based on the screening of a natural product library towards AChE and BChE inhibition we clustered the identified hits according to structural similarities into six different compound classes. Starting from this classification we investigated the compounds in-depth and selected the isoquinolines as the most promising group, especially the compound chelerythrine.

The three members of the isoquinoline group (berberine, palmatine and chelerythrine) showed selective activity towards the butyrylcholinesterase (berberine and palmatine) and activity towards both the acetyl- and butyrylcholinesterase (chelerythrine) as well as ability to prevent the aggregation of A β peptide into fibrils and to disaggregate preformed A β aggregates, the latter being very interesting as a more relevant property applicable to the clinical context. The most promising molecule was identified as chelerythrine due to its better potencies and effects on both AChE and BChE. Moreover, this compound also showed inhibition of AChE-induced A β fibril formation. For the first time, we describe a compound able to inhibit AChE and BChE, having the potency to impede self- as well as AChE-induced aggregation and possessing the capacity to disaggregate preformed A β aggregates. Recently, Niu et al. tested chelerythrine on primary peritoneal macrophages and they detected no cytotoxic activity of the compound.⁴³ In vivo treatment of SQ-20B-bearing mice (head and neck cancer) led to tumor growth delay with only minimal toxicity resulting from the treatment with chelerythrine.⁴⁴ Thus, it seems to be a well-tolerated compound. In addition, as mentioned earlier, the benzophenanthridine backbone of the chelerythrine opens routes for medicinal chemists. This scaffold provides a certain number of positions that can be chemically modified in a fast way (e.g., methoxy groups, aromatic rings, positively charged nitrogen) which is a desirable requirement to undertake future lead optimization endeavors. Altogether, these features make chelerythrine an excellent starting point in the search for more effective strategies targeting cholinergic restoration and A β fibril formation.

4. Experimental

4.1. Compound library and screening

The commercially available Enzo[®] Screen-Well[®] Natural Product Library (Enzo Life Sciences Inc., USA) consisting of 502 natural and naturally derived compounds (common names given in the Supplementary Table S1) was prepared in DMSO at a concentration of 2 mg/ml and maintained at -70°C . Prior to usage, compounds were thawed at $+37^\circ\text{C}$ in a water bath, following the manufacturer's recommendations. In order to facilitate the screening process, the natural product library was transferred to 96-well microplates using Biomek 3000 liquid handling workstation (Beckman Coulter, USA). In this step, the compounds were diluted

in Tris–HCl 50 mM pH 8 (the reaction buffer) to concentrations ranging from 1.624 to 0.117 mM. These plates were kept at +4 °C for not longer than one week, before starting the screening runs.

4.2. Exploration of the chemical space of the library

To study the chemical space occupied by the natural product library the Principal Component Analysis (PCA)-based chemical space navigation tool ChemGPS-NP was used.¹⁷ The analysis is based on 2D descriptors (total of 35) describing physical-chemical properties of the compounds and calculated from SMILES (obtained by the web-based programs ChemSpider or Molinspiration Cheminformatics v2009.01). Differences in stereochemistry were ignored as only 2D descriptors were taken into consideration. Salts, hydration information as well as counter-ions were excluded from the SMILES annotation. For analysis of the chemical space, the first four dimensions (PC1–PC4) were plotted using the software Grapher 2.1 (MacOS X, US).

4.3. Assays for measuring AChE and BChE activity

The kinetic assay used to screen for inhibition of the hydrolase activity of AChE as well as BChE is based on the Ellman's reaction¹⁴ and was carried out as described by Karlsson et al.¹⁸ The substrate concentration in both cholinesterases assay was 1.5 mM. The final concentration of the test compounds in the 96-well microplate was between 2.35 and 32.65 μ M (average concentration: 17.50 μ M) in Tris–HCl 50 mM pH 8. As control samples, 10 μ M physostigmine dissolved in methanol (reference inhibitor) or DMSO:buffer Tris–HCl 50 mM pH 8 (solvent control) were included. The starting step of the enzymatic reaction was the addition of 0.224 U/ml AChE (EC 3.1.1.7, from Electric eel, Sigma–Aldrich, USA) or 0.198 U/ml human AChE (EC 3.1.1.7, from human erythrocytes, dissolved in 0.02 M HEPES pH 8 containing 0.1% Triton X-100, Sunnyslab[®], UK) in Tris–HCl 50 mM pH 8 containing 0.1% BSA. For testing against BChE, 0.350 U/ml BChE (EC 3.1.1.8, from horse serum, Sigma–Aldrich, USA) or 0.192 U/ml recombinant human BChE (EC 3.1.1.8, expressed in transgenic goat, Sigma–Aldrich, USA) in Tris–HCl 50 mM pH 8 containing 0.1% BSA were added. Spontaneous and enzymatic hydrolysis were measured before and after enzyme addition, respectively, three times during 10 min at $\lambda = 412$ nm using a Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Finland).

4.4. Potency determination and kinetic analysis

According to the results obtained by the first screening, suitable concentration steps were selected for the active compounds to determine potency (IC_{50}) values. All compounds regarded as selective were tested against their preferential target (AChE or BChE) and unselective inhibitors were further investigated on both enzymes. In each case, potencies were calculated with at least nine different concentrations, tested in four replicates in two independent experiments. Kinetic experiments were carried out in accordance to the previously described AChE/BChE assay (Section 4.3). Chelerythrine was tested at different concentrations (0.5–2.5 μ M against AChE and 0.5–1.5 μ M against human AChE) with substrate (ATCI) concentration ranging from 0.2 to 2.0 mM. Conventional Lineweaver–Burk double-reciprocal ($1/V$ vs $1/[S]$) and Dixon ($1/V$ vs $[I]$) kinetic plots were used to identify the inhibition type and to calculate the K_i value as in Karlsson et al.¹⁸

4.5. Docking studies

Molecular docking of the compound was done using Glide module in Schrödinger package using *Torpedo californica* AChE (AChE)

crystal structure 1FSS (resolution 3 Å).⁴⁵ The protein was prepared using the Protein Preparation Wizard in Schrödinger (Schrödinger Suite 2011 Protein Preparation Wizard; Epik version 2.2, Ligprep 2.5, Glide 5.7, New York). In this wizard the bond orders are assigned, hydrogen atoms are added and atom types are defined, and the charge and protonation states are also corrected. The termini were subjected to capping and the protein was energy minimized up to 0.3 Å RMSD. All the important and conserved crystallographic water molecules were retained. The ligand structure was drawn in Maestro. The molecule was prepared using Ligprep module with MMFF force field and the grid was defined using the centroid of the active site residues. The option to dock flexibly with standard precision was selected.

4.6. Assay for measuring inhibition of A β_{1-40} aggregation using HFIP

Aggregates formation was measured by the thioflavin T (ThT) assay⁴⁶ using the A β_{1-40} peptide, which is based on the fact that in presence of A β_{1-40} aggregates, a shift in the emission and excitation spectra of ThT occurs ($\lambda_{excitation} = 385$ nm and $\lambda_{emission} = 445$ nm to $\lambda_{excitation} = 450$ nm and $\lambda_{emission} = 482$ nm).⁴⁶ The compounds were assayed using the A β_{1-40} peptide according to the protocol described by Liu et al.⁴⁷ with several modifications as indicated in Karlsson et al.¹⁸ Compounds were tested in concentrations between 1 and 250 μ M in three replicates. Immediately after adding the protein as well as after 45 min incubation at 37 °C and vigorous shaking (1000 rpm) the fluorescence was measured. As a reference compound, nordihydroguaiaretic acid,⁴⁸ dissolved in DMSO and diluted in methanol/buffer 2:1 to obtain a final concentration of 20 μ M, was used. In case of berberine, palmitate and chelerythrine the same experiments were performed without protein and the resulted values were subtracted from the data obtained with protein.

4.7. Assay for measuring disaggregation of preformed A β_{1-40} aggregates

The compounds' disaggregation behavior of preformed aggregates was measured by the ThT assay using the A β_{1-40} . The same peptide, buffers as well as procedure were used as in Section 4.6 with the following changes. First, peptide and ThT were incubated at 37 °C and vigorous shaking (1000 rpm). After 45 min the fluorescence was measured. The test compounds were added immediately in concentrations between 0.5 and 250 μ M and incubated at the same conditions for another 45 min. The reference compound, resveratrol,³⁶ was dissolved in DMSO and diluted in methanol/buffer 2:1 to obtain a final concentration of 100 μ M. The same experiments were performed without peptide and the resulted values were subtracted from the data obtained with A β .

4.8. Assay for measuring inhibition of A β_{1-40} fibril formation induced by AChE

Experiments were carried out in analogy to the procedure described by Inestrosa et al.² Immediately prior to use, an A β_{1-40} aliquot was dissolved in 25 μ l 0.1 M Tris–HCl, pH 7.4, to obtain a final concentration of 230 μ M. Stock solutions of the test compound were prepared in 0.1 M Tris–HCl, pH 7.4, and aliquotes of AChE (electric eel) were prepared in 0.1 M sodium phosphate buffer, pH 8, to achieve a final concentration of 2.3 μ M (final molar ratio protein–enzyme 100:1). The test compound was added to reach a final concentration of 1, 5, 10 and 100 μ M. Incubations were performed on a 96-well microtiter plate. To quantitate amyloid beta fibril formation, 1.5 μ M final ThT concentration in 50 mM glycine buffer pH 9.0 was added and the fluorescence was immediately

measured (excitation wavelength 440 nm, emission wavelength 485 nm) on a Thermo Scientific Varioskan Flash spectral scanning multimode reader after incubation for 24 h at 25 °C. Background fluorescence of 1.5 μ M ThT was subtracted.

4.9. Statistical analysis and data processing

In the first primary screening against both targets (AChE and BChE), each compound was tested in a single well. During the follow-up studies at least 2 replicates were used and potency determination experiments were repeated at least twice. IC₅₀ values were calculated via non-linear regression analysis (sigmoidal fitting with variable slope) using GraphPad Prism v. 4.0 (GraphPad software Inc., US). Typical statistical parameters (S/N ratio, S/B ratio, Z' factor and CV_A) to characterize the quality of screening assays were calculated for each as indicated by Karlsson et al.¹⁸

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2012.09.040>. These data include MOL files and InChIKeys of the most important compounds described in this article.

References and notes

- Rafii, M.; Aisen, P. *BMC Med.* **2009**, *7*, 7.
- Inestrosa, N. C.; Alvarez, A.; Pérez, C. A.; Moreno, R. D.; Vicente, M.; Linker, C.; Casanueva, O. I.; Soto, C.; Garrido, J. *Neuron* **1996**, *16*, 881.
- Munoz-Torrero, D. *Curr. Med. Chem.* **2008**, *15*, 2433.
- Reyes, A. E.; Perez, D. R.; Alvarez, A.; Garrido, J.; Gentry, M. K.; Doctor, B. P.; Inestrosa, N. C. *Biochem. Biophys. Res. Commun.* **1997**, *232*, 652.
- Podoly, E.; Hanin, G.; Soreq, H. *Chem. Biol. Interact.* **2010**, *187*, 64.
- Diamant, S.; Podoly, E.; Friedler, A.; Ligumsky, H.; Livnah, O.; Soreq, H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8628.
- Greig, N. H.; Utsuki, T.; Ingram, D. K.; Wang, Y.; Pepeu, G.; Scali, C.; Yu, Q. S.; Mamczarz, J.; Holloway, H. W.; Giordano, T.; Chen, D.; Furukawa, K.; Sambamurti, K.; Brossi, A.; Lahiri, D. K. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17213.
- Holmes, C.; Ballard, C.; Lehmann, D.; David Smith, A.; Beaumont, H.; Day, I. N.; Nadeem Khan, M.; Lovestone, S.; McCulley, M.; Morris, C. M.; Munoz, D. G.; O'Brien, K.; Russ, C.; Del Ser, T.; Warden, D. J. *Neurol. Neurosurg. Psychiatry* **2005**, *76*, 640.
- Bolognesi, M. L.; Cavalli, A.; Bergamini, C.; Fato, R.; Lenaz, G.; Rosini, M.; Bartolini, M.; Andrisano, V.; Melchiorre, C. *J. Med. Chem.* **2009**, *52*, 7883.
- Mangialasche, F.; Solomon, A.; Winblad, B.; Mecocci, P.; Kivipelto, M. *Lancet Neurol.* **2010**, *9*, 702.
- Bolognesi, M. L.; Matera, R.; Minarini, A.; Rosini, M.; Melchiorre, C. *Curr. Opin. Chem. Biol.* **2009**, *13*, 303.
- Howes, M.-J. R.; Perry, E. *Drugs Aging* **2011**, *28*, 439.
- Zaid, H.; Raijn, J.; Nasser, A.; Saad, B.; Rayan, A. *Open Nutraceuticals J.* **2010**, *3*, 194.
- Ellman, G. L.; Courtney, K. D.; Andres, V.; Feather-Stone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.
- Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. *J. Biomol. Screen.* **1999**, *4*, 67.
- Chen, X.; Tikhonova, I. G.; Decker, M. *Bioorg. Med. Chem.* **2011**, *19*, 1222.
- Larsson, J.; Gottfries, J.; Muresan, S.; Backlund, A. *J. Nat. Prod.* **2007**, *70*, 789.
- Karlsson, D.; Fallarero, A.; Brunhofer, G.; Guzik, P.; Prinz, M.; Holzgrabe, U.; Erker, T.; Vuorela, P. *Eur. J. Pharm. Sci.* **2012**, *45*, 169.
- Karlsson, D.; Fallarero, A.; Brunhofer, G.; Mayer, C.; Prakash, O.; Mohan, C. G.; Vuorela, P.; Erker, T. *Eur. J. Pharm. Sci.* **2012**, *47*, 190.
- Comprehensive Natural Products II Chemistry and Biology*; Backlund, A., Lui, L. M. H., Eds.; Elsevier: Oxford, UK, 2010; Vol. 3, p 47.
- Decker, M.; Kraus, B.; Heilmann, J. *Bioorg. Med. Chem.* **2008**, *16*, 4252.
- Tian, Y.; Bustos, V.; Flajolet, M.; Greengard, P. *FASEB J.* **1934**, *2011*, 25.
- Pereira, D. M.; Ferreres, F.; Oliveira, J. M. A.; Gaspar, L.; Faria, J.; Valentão, P.; Sottomayor, M.; Andrade, P. B. *Phytomedicine* **2010**, *17*, 646.
- Ferry, N.; Goodhardt, M.; Hanoune, J.; Sevenet, T. *Br. J. Pharmacol.* **1983**, *78*, 359.
- Shimada, Y.; Goto, H.; Itoh, T.; Sakakibara, I.; Kubo, M.; Sasaki, H.; Terasawa, K. *J. Pharm. Pharmacol.* **1999**, *51*, 715.
- Yuzurihara, M.; Ikarashi, Y.; Goto, K.; Sakakibara, I.; Hayakawa, T.; Sasaki, H. *Eur. J. Pharmacol.* **2002**, *444*, 183.
- Wang, L.; Li, W.; Liu, Y. *Pharmazie Int. J. Pharm. Sci.* **2008**, *63*, 606.
- Darvesh, S.; McDonald, R. S.; Darvesh, K. V.; Mataija, D.; Conrad, S.; Gomez, G.; Walsh, R.; Martin, E. *Bioorg. Med. Chem.* **2007**, *15*, 6367.
- Carolan, C. G.; Dillon, G. P.; Khan, D.; Ryder, S. A.; Gaynor, J. M.; Reidy, S.; Marquez, J. F.; Jones, M.; Holland, V.; Gilmer, J. F. *J. Med. Chem.* **2010**, *53*, 1190.
- Dillon, G. P.; Gaynor, J. M.; Khan, D.; Carolan, C. G.; Ryder, S. A.; Marquez, J. F.; Reidy, S.; Gilmer, J. F. *Bioorg. Med. Chem.* **2010**, *18*, 1045.
- Takadoi, M.; Yamaguchi, K.; Terashima, S. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3271.
- Bubser, M.; Byun, N.; Wood, M. R.; Jones, C. K.; Fryer, A. D.; Christopoulos, A.; Nathanson, N. M., Eds.; Muscarinic Receptor Pharmacology and Circuitry for the Modulation of Cognition Muscarinic Receptors. Springer: Berlin, Heidelberg, 2012; Vol. 208, p 121.
- Shan, W. J.; Huang, L.; Zhou, Q.; Meng, F. C.; Li, X. S. *Eur. J. Med. Chem.* **2011**, *46*, 5885.
- Catto, M.; Aliano, R.; Carotti, A.; Cellamare, S.; Palluotto, F.; Purgatorio, R.; De Stradis, A.; Campagna, F. *Eur. J. Med. Chem.* **2010**, *45*, 1359.
- Cohen, T.; Frydman-Marom, A.; Rechter, M.; Gazit, E. *Biochemistry* **2006**, *45*, 4727.
- Feng, Y.; Wang, X. P.; Yang, S. G.; Wang, Y. J.; Zhang, X.; Du, X. T.; Sun, X. X.; Zhao, M.; Huang, L.; Liu, R. T. *Neurotoxicology* **2009**, *30*, 986.
- Ortega, A.; Rincón, Á.; Jiménez-Aliaga, K. L.; Bermejo-Bescós, P.; Martín-Aragón, S.; Molina, M. T.; Csáky, A. G. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2183.
- Dinamarca, M. C.; Cerpa, W.; Garrido, J.; Hancke, J. L.; Inestrosa, N. C. *Mol. Psychiatry* **2006**, *11*, 1032.
- Moss, M. A.; Varvel, N. H.; Nichols, M. R.; Reed, D. K.; Rosenberry, T. L. *Mol. Pharmacol.* **2004**, *66*, 592.
- Xie, W.; Stribley, J. A.; Chatonnet, A.; Wilder, P. J.; Rizzino, A.; McComb, R. D.; Taylor, P.; Hinrichs, S. H.; Lockridge, O. J. *Pharmacol. Exp. Ther.* **2000**, *293*, 896.
- Bartolini, M.; Bertucci, C.; Cavrini, V.; Andrisano, V. *Biochem. Pharmacol.* **2003**, *65*, 407.
- Rouleau, J.; Iorga, B. I.; Guillou, C. *Eur. J. Med. Chem.* **2011**, *46*, 2193.
- Niu, X. F.; Zhou, P.; Li, W. F.; Xu, H. B. *Fitoterapia* **2011**, *82*, 620.
- Chmura, S. J.; Dolan, M. E.; Cha, A.; Mauceri, H. J.; Kufe, D. W.; Weichselbaum, R. R. *Clin. Cancer Res.* **2000**, *6*, 737.
- Harel, M.; Kleywegt, G. J.; Ravelli, R. B.; Silman, I.; Sussman, J. L. *Structure* **1995**, *3*, 1355.
- LeVine, H. *Protein Sci.* **1993**, *2*, 404.
- Liu, R.; Yuan, B.; Emadi, S.; Zameer, A.; Schulz, P.; McAllister, C.; Lyubchenko, Y.; Goud, G.; Sierks, M. R. *Biochemistry* **2004**, *43*, 6959.
- Ono, K.; Hasegawa, K.; Naiki, H.; Yamada, M. *Biochim. Biophys. Acta* **2004**, *1690*, 193.
- Okmanov, R.; Tozhiboev, A.; Turgunov, K.; Tashkhozhaev, B.; Khakimova, Z.; Tulyaganov, T.; Shakhidoyatov, K. *J. Struct. Chem.* **2009**, *50*, 1149.
- Tulyaganov, T.; Ibragimov, A. *Chem. Nat. Compd.* **1993**, *29*, 512.
- Wu, L. X.; Gu, X. F.; Zhu, Y. C.; Zhu, Y. Z. *Eur. J. Pharmacol.* **2011**, *650*, 290.
- Martinez, P. J. A.; Rodriguez, A. M. R.; Biart, M. M. T.; Machua, V. M. *Rev. Cubana Farm.* **1987**, *21*, 165.
- Samuelsson, G.; Bohlin, L.; Drugs of Natural Origin. Swedish Pharmaceutical Press: Stockholm, 2009. Chapter 10.
- Samikov, K.; Shakirov, R.; Yunusov, S. *Chem. Nat. Compd.* **1977**, *13*, 559.
- Zhu, H. J.; Wang, J. S.; Guo, Q. L.; Jiang, Y.; Liu, G. Q. *Biol. Pharm. Bull.* **1974**, *2005*, 28.
- Rohrer, U.; Kunz, E. M.; Lenkeit, K.; Schaffner, W.; Meyer, J. *Schweiz. Monatsschr. Zahnmed.* **2007**, *117*, 1126.
- Wong, L. S.; Sharp, L. A.; Xavier, N. M.; Turner, P.; Sherburn, M. S. *Org. Lett.* **1955**, *2002*, 4.
- Ryuk, J. A.; Zheng, M. S.; Lee, M. Y.; Seo, C. S.; Li, Y.; Lee, S. H.; Moon, D. C.; Lee, H. W.; Lee, J. H.; Park, J. Y.; Son, J. K.; Ko, B. S. *Arch. Pharm. Res.* **2012**, *35*, 1045.
- Miao, F.; Yang, X. J.; Zhou, L.; Hu, H. J.; Zheng, F.; Ding, X. D.; Sun, D. M.; Zhou, C. D.; Sun, W. *Nat. Prod. Res.* **2011**, *25*, 863.