Tacrines as Therapeutic Agents for
Alzheimers Disease. V. Recent
Developments

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Abstract: Herein we have reviewed our recent developments for the identification of new tacrine analogues for Alzheimer's disease (AD) therapy. Tacrine, the first cholinesterase inhibitor approved for AD treatment, did not stop the progression of AD, producing only some cognitive improvements, but exhibited secondary effects mainly due to its hepatotoxicity. Thus, the drug was withdrawn from the clinics administration. Since then, many publications have described non-hepatotoxic tacrines, and in addition, important efforts have been made to design multitarget tacrines by combining their cholinesterase inhibition profile with the modulation of other biological targets involved in AD.

Keywords: Alzheimer's disease, Cholinesterase inhibition, Friedländer reaction, Hepatotoxicity, Tacrine derivatives

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, and the most common cause of dementia in the elderly, against which there is no effective treatment to date.^[1–4] In the early 1970s, research was aimed at understanding the biochemical mechanisms involved in AD, establishing the rational basis for the development of a therapy.^[5] It was discovered that in AD, the neocortical deficiency of choline acetyltransferase, the enzyme responsible for the synthesis of acetylcholine (ACh), together with the reduction of choline absorption, the decrease in the concentration of ACh by hydrolysis of cholinesterase enzymes (ChEs), the loss of cholinergic neurons of the Meynert basal nucleus and the reduction in the number of nicotinic and muscarinic ACh receptors, were clear indications that in AD there was a loss of cholinergic transmission, and that these biological processes were linked to the deterioration of the cognitive abilities of the AD patients,^[6–10] setting the principles of the *cholinergic hypothesis* of AD.

By using synaptic theoretical approaches for central cholinergic enhancement, Summers^[11] reported a pilot study in twelve patients with tacrine (1,2,3,4-tetrahydroacridin-9-amine), followed by a successful long-term treatment to evaluate the ability of tacrine to improve cognitive defects in

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AD patients. As a result, the first AChE inhibitor (AChEI) was approved for the treatment of AD, launched to the market under the trade name of Cognex.^{*} However, tacrine was later withdrawn due to its hepatotoxic effects, as nearly half of the patients had increased serum alanine aminotransferase, and a smaller percent had severe nausea and vomiting. A second generation of ChEIs was developed in order to avoid the side effects of tacrine.^[12]

There are currently three ChEIs approved by the US Food and Drug Administration (donepezil, rivastigmine and galantamine), but these drugs neither cure nor stop the progression of AD. Recent studies showed that donepezil has a promising disease-modifying potential due to a decrease of hippocampal atrophy in patients with mild cognitive impairment, compared to placebo.^[13] Therefore, a renewed interest for ChEIs, not only for managing the symptoms, but also as promising disease-modifying drugs in AD, was observed, and as a consequence, for new ChEIs based on tacrine with better Absorption Distribution Metabolism Excretion-Toxicity (ADME-Tox) properties.

Very interestingly, AChEIs binding at the Peripheral Anionic Site (PAS) are known to play an important role inhibiting amyloid beta (A β) aggregation induced by AChE. In a study carried out by Inestrosa and co-workers^[14] the aggregation of the A β peptide was simulated for 24 h, and the amount of the aggregated peptide was quantified by measuring the remaining amount of soluble peptide after incubation using a sedimentation test. The addition of AChE led to a 3-fold increase in A β _{1–40} peptide aggregation compared to the experiment where the peptide was used alone. These tests were carried out using the thioflavin T (ThT) aggregation assay. The molar ratio between A β _{1–40} and AChE was 100/1 and the test demonstrated that AChE was a A β _{1–40} aggregation promoter. This study found that AChEIs preferentially binding at the PAS had a greater effect in inhibiting A β aggregation.

On the other hand, although the etiology of AD has not yet been elucidated, AD is a multifactorial disease, involving several co-occurring processes. Therefore, using a strategy focused on a single drug target is not satisfactory, and the reason why the current efforts to find a therapy for AD are focused on multitarget small molecules (MSM) to increase the modulation of multiple biological targets, that may improve both therapeutic safety and efficacy.^[15–19]

Our research group has been developing new MSM for AD, based on tacrines designed by incorporating diverse pharmacophores, with the aim to obtain potential drugs with better ADME-Tox properties. These progresses and results have been reviewed and updated.^[20–23] Continuing with this effort, in this account we have reviewed the most recently results carried out in our laboratories during the last decade in the frame of the design and synthesis of non-hepatotoxic

tacrines, targeted to bind at specific AChE sites, as single tacrine monomers, or as bivalent inhibitors simultaneously binding at the AChE catalytic anionic site (CAS) and PAS.

2. Catalytic AChE Site Targeted Tacrines

A large number of tacrines have been obtained by Lewis acid catalyzed modified Friedländer reaction (FR), starting from suitable *o*-aminonitriles and cycloalkanones. Originally, FR was the acid or base promoted condensation of an aromatic 2-amino-substituted carbonyl compound with an appropriately substituted carbonyl derivative containing an reactive α -methylene group, followed by cyclodehydration.^[24] *o*-Aminonitriles could be installed in aromatic heterocyclic such as quinolines, pyrimidines, quinazolines, quinazolinones, quinazolinidiones and other fused nitrogen-containing heterocycles that could have important pharmacological interest. Thus, we have designed new tacrine analogues in which the central core 4-aminopyridine, essential for the binding at the CAS of AChE, was maintained, replacing the benzene ring A with a pyridine, quinone, pyran, quinoxaline, and quinoline structural and functional motifs (Figure 1).

2.1. Pyridotacrines

Pyridotacrines (PT, **I**)^[25] (Figure 1) are tacrine analogues bearing the 1,8-naphthyridine motif, whose design was inspired by other tacrines that did not present hepatotoxic effects compared to tacrine, such as tacripyrines, a series of potent inhibitors of ChEs, binding preferentially at the PAS of AChE and therefore able to inhibit A β aggregation, and Ca²⁺ channel blockers,^[26] or the 2-aminopyridine-3,5-dicarbonitriles

Figure 1. Catalytic AChE site designed tacrines.

and 2-chloropyridine-3,5-dicarbonitrile, which presented moderate activity against ChEs and neuroprotection,^[27] and were used as precursors for the synthesis of **PT1-12** (Table 1).

From the IC_{50} inhibition values (Table 1), it was apparent that all tacrines bearing a chlorine atom at C-2 were poor AChEI, a result in good agreement with the fact that pyridoctacrines bearing electron donating groups at position C-2 showed inhibition of *EeAChE* in the nanomolar range.

In this study, the authors identified tacrine **PT1** (Table 1), as a very potent and selective AChEI, in the nanomolar range. Note that tacrine **PT5** (Table 1) only differing from tacrine

PT1 in the phenyl group at C-4, was less potent than **PT1**. In spite that tacrine **PT6** (Table 1) [IC_{50} (*EeAChE*) = 0.06 ± 0.01 μ M; IC_{50} (*eqBuChE*) = 9.6 ± 0.6 μ M] is a less potent AChEI than **PT1**, ligand **PT6** was obtained in higher chemical yield, exhibited better balance between potency and selectivity, and an inhibition power against *EeAChE* (K_i = 69.2 nM) in the same order of magnitude as **PT1**, showing a non-competitive *EeAChE* inhibition mechanism.

Molecular modeling showed that tacrines **PT1-12** were almost planar compared to tacipyridines,^[26] which have an angular geometry because the dihydropyridine ring is bent compared to the pyridine ring of compounds **PT1-12**. This may explain why these tacrines can enter without restriction and impediment through the binding pocket until getting the CAS of AChE, and therefore not binding at the PAS of AChE. The binding mode of tacrine **PT6** at *EeAChE* was determined by means of a molecular modelling study (Figure 2), using AutoDock 4.0 software,^[28] confirming that this ligand, as expected, and similarly to tacrine, binds at the CAS of AChE (Figure 2).

Finally, the neuroprotection^[29] capacity of tacrines **PT1-12** (Table 1) was determined by using a reactive oxygen species generation model (ROS), based on the cocktail of two mitochondrial respiratory chain blockers, rotenone and oligomycin (R/O), and the hyperphosphorylation model of the tau protein using okadaic acid (OA), a well-known phosphatase inhibitor protein. From these results it was concluded that the neuroprotective profile of these tacrines was moderate, the most potent being tacrines **PT2** and **PT7** (Table 1), which reduced cell death around 29 %. Alike, a study where neuronal death was induced using OA was carried out, showing the effect of **PT6** (Table 1) to block or rescue neurons from brain death.

To sum up, tacrines **PT1-12** (Table 1) can be considered attractive therapeutic molecules playing key role in cholinergic dysfunction and oxidative stress in the progression of AD.

2.2. Quinonepyranotacrines

Racemic quinonepyranotacrines (**QPT**, **II**) are tacrine analogues designed by juxtaposition of tacrine and 1,4-naphthoquinone (Figure 1), both motifs linked by a 4*H*-pyrane ring, bearing two different electron withdrawing groups (NO_2 and F) and an electron donating group (OMe) in the aromatic ring located at the central pyran ring. Ten members of this family (**QPT1-10**) (Figure 3) were prepared from easily available precursors in good overall yield.^[30]

Possible **QPTs** hepatotoxicity assay was performed using the human hepatocellular carcinoma cell line (HepG2).^[29] This cell line possesses many genetic characteristics, typical of normal liver cells and is an alternative to hepatocytes. HepG2 cells are used for the experimental evaluation of the potential

Table 1. Synthesis and ChE inhibition of **PT1-12**.

PT	R	X	Y	<i>EeAChE</i> IC_{50} (μ M)	<i>eqBuChE</i> IC_{50} (μ M)
1	H	Me ₂ N	CH ₂	0.014 ± 0.001	5.2 ± 0.4
2	H		CH ₂	0.043 ± 0.006	5.6 ± 0.3
3	H		CH ₂	0.025 ± 0.005	3.6 ± 0.2
4	H		CH ₂	0.030 ± 0.003	2.6 ± 0.3
5	Ph	Me ₂ N	CH ₂	0.09 ± 0.02	7.5 ± 0.1
6	H	MeO	CH ₂	0.06 ± 0.01	9.6 ± 0.6
7	H	EtO	CH ₂	0.050 ± 0.001	> 30
8	Ph	MeO	CH ₂	0.08 ± 0.01	5.1 ± 0.9
9	H	Cl	CH ₂	5.0 ± 0.4	> 30
10	Ph	Cl	CH ₂	> 30	> 30
11	H	MeO	NBn	0.35 ± 0.04	> 100
12	H	Cl	NBn	0.35 ± 0.09	> 100

Figure 2. Binding mode of **PT6** at the CAS of AChE.

Figure 3. General structure of Quinopyranotacrine (II).

metabolic changes that the liver cells would undergo under the influence of a drug. The use of the HepG2 line allows studying the action of hepatotoxins and drugs, using a multitude of parameters, such as cell viability, proliferation, membrane integrity, to assess toxicity. In this case, an *in vitro* cell viability test was carried out at five different concentrations (10, 30, 100, 300 and 1000 μ M), using tacrine as a reference. For most **QPTs**, at 1000 μ M dose, no significant toxicity was observed, indicating that **QPTs** are non-hepatotoxic tacrines.

Next, the assessment of the inhibition of ChEs was carried out using the Ellman's test.^[31] **QPT1-10** showed from moderate to poor human ChE (hChE) inhibition, the most potent being **QPT4** (Figure 3) [IC_{50} (hChE) = 1.10 ± 0.15 μ M], selective against hBuChE, and 3.5-fold less hepatotoxic than tacrine at 1000 μ M. The kinetic analysis of hAChE inhibition for **QPT4** showed that the Lineweaver-Burk graph^[32] fits into a mixed-type inhibitor behavior with K_i value of 3.64 ± 2.06 μ M.

The antioxidant activity was determined by measuring the Oxygen Radical Absorbance Capacity (ORAC test)^{[33][34]}. The results were expressed as Trolox/equivalent (TE), indicating the ability of the tested compounds to scavenge peroxy radicals produced in the test, ferulic acid being used as a positive control. None of the **QPTs** were more potent than ferulic acid, and according to the structure-activity relationship of the compounds, it was observed that those with electron-withdrawing groups in the *para*- position of the phenyl group presented the highest antioxidant activity, the most potent antioxidant being **QPT10** (3.34 ± 0.14 TE).

Next, a very detailed docking study the most promising **QPT4**, using AutoDock Vina software^[28] was carried out, in which the docking of each of the enantiomers in hAChE was analyzed. The process of recognizing each of the **QPT4** enantiomers with hAChE was done using a novel docking method that considers some key amino acid residues flexible, allowing hAChE to adjust its conformation to the substrate keeping the rest of the enzyme rigid. (*R*)-**QPT4** docking revealed several non-covalent interactions that play an important role in the interaction of **QPT4** and the binding pocket of hAChE (Figures 4a-c). A hydrogen bond interaction between the carbonyl oxygen from quinone and the N-H of Phe 295 was observed, likewise hydrophobic π - π stacking interactions between Tyr341 and Trp286 with the fused aromatic systems of the (*R*)-**QTP4** molecule was also highlighted (Figure 4c). Additionally, *p*-methoxybenzene forms a π - π Edge to phase interaction with Tyr341. On the other hand (*S*)-**QPT4** interacts with the PAS of hAChE in a similar way that *R* enantiomer (Figure 4b). The quinone motif facilitates π - π stacking interactions with the Trp286 side chain of PAS, likewise the pyridine and the *p*-methoxybenzene fragments provide π - π stacking interaction with Trp341. Additionally, the nitrogen of the pyridine facilitates a hydrogen bond interaction with the carboxylate group of Asp74 (Figure 4d). The most stable conformations generated through docking show that both enantiomers occupy the same spatial region of the PAS from hAChE, but in an inverse orientation, as can be seen in Figures 4a and 4b. However, the binding energy of both **QPT4** enantiomers reveal that the *R* enantiomer (-12.4 Kcal/mol) exhibits lower binding energy than *S* enantiomer (-10.4 Kcal/mol) when they interact with the binding site of hAChE.

Lastly, ADME-Tox calculations were carried out with the aim of measuring pharmacokinetic properties of all enantiomers of **QPTs**. Overall, most of molecules presented optimal pharmacokinetic properties and fit well with what the theory predicts for drugs that act in the CNS, however, **QPT4** (Figure 3) presented a Polar Surface Area (PSA) value of approximately 100 $\text{\AA}^2$ for each enantiomer. The PSA value for drugs that are going to act in the CNS should not exceed 60 $\text{\AA}^2$. Likewise, this study evaluated the ability of **QPT1-10** to cross the blood-brain barrier (BBB), a fundamental criterion that a drug should meet in order to act in the central nervous system (CNS), showing that **QPT4** was predicted to cross the BBB, being permeable with a penetration estimation (Pe) of 4.4 ± 0.9 , this value being within the Pe range of tacrine and donepezil, two reference drugs known for their ability to cross the BBB.

Figure 4. Binding mode of the inhibitors (*R*)-QPT4 and (*S*)-QPT4 within the active site of hAChE. a) (*R*)-QPT4 is rendered as ball and sticks (carbon atoms in purple) b) (*S*)-QPT4 is rendered as ball and sticks (carbon atoms in dark blue). The side chain conformations of the mobile residues are illustrated in the same light color as the ligand, catalytic triad residues (CT) in green, oxanion hole residues (OH) in magenta, anionic sub-site (AS) in orange, except Trp86, acyl binding pocket (ABP) in yellow and PAS in the same color as flexible residues. c) 2D interaction map of compound (*R*)-QPT4 d) 2D interaction map of compound (*S*)-QPT4.

2.3. Quinoxalinetacrines

The aim of this study was the synthesis and biological evaluation of Quinoxalinetacrines (QTs, **III**) (Figure 1) as new non-hepatotoxic and neuroprotective tacrine analogues, resulting from the juxtaposition of quinoxaline and tacrine, and

particularly the analysis of QT78 (Scheme 1), the most simple member of this family.^[35]

The synthesis of QT78 has been carried out by the reaction of 3-amino-2-quinoxalinecarbonitrile and cyclohexanone, using Lewis acid AlCl_3 as catalyst, under typical FR conditions, in 88% yield (Scheme 1).

Scheme 1. Synthesis of QT78.

The *in silico* study of the toxicity^[36] showed that there was not enough evidence to predict an adverse outcome of the toxicity of QT78 and its metabolites. However, a cell viability study was performed in the HepG2 cell line to measure *in vitro* hepatotoxicity of QT78. The results showed that at high concentrations of QT78 (300 to 1000 μM) cell viability decreased, but hepatotoxicity was significantly lower compared to tacrine, which showed a decrease in cell viability even at much lower concentrations (30 μM).

Regarding the inhibition of ChEs, QT78 was a modest but selective inhibitor of BuChE [IC_{50} (hAChE) = 22.0 ± 1.3 μM , and IC_{50} (hBuChE) = 6.79 ± 0.33 μM] and less potent than tacrine.

The docking study on AChE [28] showed that QT78 (Figure 5a) fits well in the middle of the active site gorge without interacting with the catalytic triad amino acids. The cyclohexane ring is directed towards the bottom of the gorge, establishing π -alkyl interaction with Trp86. The target-ligand complex is also stabilized by molecular interactions with some amino acid residues of the PAS. Likewise, a hydrogen bond network is established between the amino group and pyrazine nitrogen ring from QT78 with Tyr124 and Asp74. Additionally, the phenyl ring forms π - π stacking interaction with Tyr341. Nevertheless, the docking with hBuChE showed that QT78 fits inside of CAS (Figure 5b), but it does not occupy the same space as tacrine. According to the docking data, the amino group of QT78 forms two hydrogen bond interactions with the catalytic triad residues His438 and Ser198. In addition, the pyridine and cyclohexane rings interact with residues in the acyl binding pocket (ABP) Phe329 and Leu286. All these molecular modeling results on the two enzymes are complemented with experimental inhibition in ChEs shown by QT78 as a moderate hBuChE selective ChEI.

In silico analysis of the pharmacokinetic properties of QT78 gave a satisfactory result based on the values of the Lipinski's rule for drugs that act on the CNS.^[37] It should be bore in mind that Lipinski's rule values for drugs acting on the CNS are more restricted compared to the other drugs that do not act on the CNS. In the case of QT78, these values are molecular weight < 450 g mol^{-1} , hydrogen bond donor < 3, and hydrogen bond acceptor < 7. Besides, the aqueous solubility (QLogS = -3.746) is between (-6.5 to -0.5), while the partition coefficient (QLogPo/W = 2.534) indicates

Figure 5. Molecular modeling for compound QT78. a) Binding mode of QT78 in AChE binding-site. Ligand is rendered as ball and sticks (carbon atoms in pink) and the side chain conformations of the mobile residues are illustrated in light pink, CT in green, OH in magenta, AS in orange, except Trp86, ABP in yellow and PAS in light pink. b) Binding mode of QT78 (orange) and tacrine (cherry) within the BuChE binding-site. Catalytic anionic site (CAS) in green, OH in red, choline binding site in violet (CBS), ABP in yellow, and PAS in blue. Hydrogen bonding interactions were showed in dashed lines.

a good absorption of QT78 in the body since it is in the optimal range of (-2.0 to -6.5). QPLogBB value is -0.332 which would indicate that the QT78 could easily penetrate the BBB since its optimal range is (-2.0 to 1.04). Another important value to take into account is the PSA, which is defined as the area of a molecule's van der Waals surface that arises from the oxygen, nitrogen or hydrogen atom attached to oxygen or nitrogen atoms. For drugs that are going to act in the CNS, PSA must not exceed $60 \text{ \AA}^2$. In the case of QT78 this value is $59.549 \text{ \AA}^2$. PSA is commonly used in medicinal chemistry as a metric for the optimization of cell permeability. Molecules with a PSA greater than $140 \text{ \AA}^2$ are usually believed to be poor at permeating the cell membrane.

Finally, regarding the neuroprotection, QT78 produced a significant increase in cell viability against R/O, 16.7% and 21.4%, respectively, and a powerful increase in cell viability against OA (18.9%). In all cases, when increasing the

concentration of the compound to 1 μM , the protective capacity decreased against all the toxic stimuli tested.

To sum up, in the search for new non-hepatotoxic tacrines for AD, **QT78** was identified as a promising compound, as it is less toxic than tacrine at high concentrations (100 μM to 1 mM), a selective BuChEI, exhibiting significant neuro-protective effect against different toxic stimuli, including ROS generation and hyperphosphorylation of tau protein.

2.4. (Benz)imidazopyridinotacrines

(Benz)imidazopyridinotacrines (**IV**) (Figure 1) are two new families of tacrines designed by incorporating a 2-(1-methyl-1*H*-imidazolyl) (**IVa**) (Figure 6), or 2-(1-methyl-1*H*-benzimidazolyl) (**IVb**) (Figure 6) at C8, and a cycloalkane at C-6/C-7, into tacrine scaffold, and prepared from readily available precursors using the FR to build the tacrine motif.^[38]

These new tacrine analogues were evaluated by analysing their cytotoxicity in human hepatocellular carcinoma cells and measuring the inhibition of ChEs, along with their antioxidant capacity.

The new tacrines were in general non-hepatotoxic at high doses, potent and selective *EeAChE*s, but poor antioxidant agents. Among them, 12-(1-methyl-1*H*-imidazol-2-yl)-2,3,4,7,8,9,10,11-octahydro-1*H*-benzo[*b*]cyclohepta[*g*][1,8]naphthyridin-13-amine (**IPT**) (Figure 6) is non-hepatotoxic and selective *EeAChE* inhibitor (IC_{50} *EeAChE* = 0.50 ± 0.03 μM).^[38]

2.5. Kojotacrines

Racemic kojotacrines (**KTs**, **V**, Figure 1) are new tacrine analogues designed by combining antioxidant kojic acid (KA) and tacrine, in our search for new non-hepatotoxic tacrines for AD.^[39] Therefore, the known properties of tacrine and the recognized antioxidant characteristics of KA as a natural fungal metabolite able to sequester ROS, endowed the new **KT** hybrids with potential ChE inhibition plus antioxidant capacity.

The synthesis of these **KTs** was carried out in two steps using the readily available KA, that was reacted with a selection

of arylidenemalonitriles in the presence of trimethylamine, in good yields, followed by FR with cyclohexanone, under the usual experimental conditions resulting in the expected racemic mixture.^[39] **KTs** were substituted at different positions, with diverse type of substituents, at the aromatic ring.

The biological evaluation of these **KTs** included: (1) *In vitro* cytotoxicity in HepG2 cells, by analysis of the cell viability at different concentrations; most of the **KTs** are less hepatotoxic than tacrine at 1000 μM , and among them, **KT1** (Figure 7) was 6-fold less hepatotoxic than tacrine. (2) The inhibition of *EeAChE* and *eqBuChE*; all the **KTs** are moderate, but selective *AChE*s, less potent than tacrine, **KT2** being the most potent *EeAChE* ($\text{IC}_{50} = 0.64 \pm 0.006$ μM), 20-fold less potent than tacrine. (3) The evaluation of antioxidant capacity; **KT1** and **KT3** (Figure 7) bearing a methoxy group at C2, and two chlorine atoms at positions C2 and C6, respectively (Figure 7), were the most powerful antioxidants [$\text{R} = 2\text{-OCH}_3$ (6.05 ± 0.41 TE) and $\text{R} = 2,6\text{-di-Cl}$ (6.14 ± 0.40 TE)]. However, **KT2** was the most balanced **KT** considering its hepatotoxicity, neuroprotection, cytotoxicity and ChE inhibition. (4) Neuroprotection. Cell viability of neuroblastoma SH-SY5Y cells treated with the different concentration of **KTs** incubated for 24 h was, showed that **KT2** had similar neuroprotection against $\text{A}\beta_{1-40}$ insult at 3 and 10 μM , being less neuroprotective than KA.

Taking into account these results, **KT2** was selected for docking analysis using Autodock Vina to justify its binding properties at hAChE. Molecular modeling was carried out on the two enantiomers of **KT2** (Figure 8). For compound (**R**)-**KT2**, an hydrogen bond was established between Tyr124-OH and the nitrogen atom of the pyranotacrine portion (Figure 8a). For compound (**S**)-**KT2**, the amino group establishes a hydrogen bond interaction with the Asp74 carboxylate group (a key residue of PAS), and the oxygen atom of the methoxy group establishes a hydrogen bond interaction with the main chain -NH- group of Thr75 (Figure 8b). As shown in

Figure 6. Structure of (benz)imidazopyridinotacrines (**IVa**, **b**) and tacrine **IPT**.

Figure 7. Structure of **KT1-3**.

Figure 8. Docking pose of inhibitors (*R*)-**KT2** and (*S*)-**KT2** at the active site of hAChE. a) (*R*)-**KT2** is rendered as ball and sticks (carbon atoms in pink) b) (*S*)-**KT2** is rendered as ball and sticks (carbon atoms in blue). Hydrogen bonds were showed in black dashed lines.

Figure 8, both enantiomers display hydrogen bond interactions with residue Tyr72 and are positioned on the active site of the enzyme in a manner that interacts with amino acids at the edge of the cavity, they act as a barrier to the substrate for entering the active site.

In silico analysis of ADME-Tox properties was carried out using the QikProp module of Schrodinger suite in order to evaluate the drug-likeness of the synthesized **KTs**. The calculated ADME-Tox properties were in line with the parameters required by the Lipinski's rule for drugs to be used for the CNS. However, molecule **KT2** had a PSA of 107 Å² for each enantiomer, much higher than the required value of less than 60 Å² required for drugs acting in the CNS.

Overall, ligand **KT2** is a moderate *EeAChI* in the submicromolar range, presenting neuroprotective and antioxidant activity, 6-fold less hepatotoxic than tacrine, and consequently, a potential new hit for further investigation for AD therapy.

3. Bivalent Tacrines

New and potent AChEIs have been identified by using the “bivalent ligand strategy”, that consists of the design of single ligands able to bind simultaneously the CAS and PAS of AChE.^[40,41] In the first reported example, for instance, two tacrine units were linked by a seven methylene spacer to bind both the CAS and PAS, resulting in the bivalent AChEI bis (7)-tacrine, which showed enhanced affinity toward AChE compared to tacrine.^[42]

3.1. Tacrine-ferulic Acid Hybrids

As mentioned before, the drug discovery approach for AD therapy based on the MSM^[15,16] strategy involves the incorporation of different pharmacophores from various reference molecules in the same structure to obtain a hybrid molecule, where each pharmacophore retains its capacity for acting in a specific manner and producing the desired pharmacological response, or even better, generate a synergetic effect as a result of the combination of these active components. Accordingly, multicomponent reactions,^[43] such as Ugi reaction,^[44] are perfect for the MSM strategy, since several active fragments can be coupled in a single reaction and form a unique multipotent molecule.

By following the MSM strategy for AD, tacrine-ferulic acid hybrids (**VI**, **TEAHs**) (Figure 9)^[17] were designed showing strong antioxidant power, reversible non-competitive AChE inhibition, being capable of binding both PAS and CAS.

Figure 9. Bivalent tacrine hybrids **VI**-**VIII**.

These compounds bear a substituted glycine substructure as part of the main structure of α -acylamino-carboxamide, tacrine or 7-methoxytacrine motifs and an antioxidant feruloyl group, both attached to the glycine fragment. The carbonyl group of the glycine moiety appears as a *N*-benzylamide, 2'-chloro-6'-methylaniline, or 2'-naphthylamine, functional groups that should provide the ChE inhibitory activity, binding at the hydrophobic PAS of AChE, with possible consequences on A β aggregation.

The synthesis of the new tacrines has been carried out starting from readily available precursors, using the Ugi reaction, which involves the condensation of four reaction components (aldehyde, amine, carboxylic acid, isocyanide).^[44] Thus, this reaction would allow the rapid one-step synthesis of α -aminoacyl amide derivatives containing different fragments, incorporating for instance, neuroprotective, antioxidant properties, along with capacity of inhibiting ChEs and the aggregation of A β _{1–42}, important biological targets in the fight against AD.

As a result of the biological evaluation, the authors identified **TFAH1** (Figure 9) as a particularly interesting multipotent compound that shows moderate and completely selective inhibition of hBuChE (IC_{50} = 68.2 nM), strong antioxidant activity (4.29 ± 0.19 TE), good A β anti-aggregation properties ($65.6 \pm 0.9\%$ at 1:1 ratio), able to cross BBB, as determined by PAMPA-BBB assay, low hepatotoxicity ($59.4 \pm 4.7\%$ cell viability at 1000 μ M), good neuroprotection against toxic insults such as A β _{1–40}, A β _{1–42}, H₂O₂, and O/R on SHSY5Y cells, at 1 μ M.

It was clear that **TFAH** hybrids can act as particularly interesting multipotent compounds for the potential AD therapy.

3.2. Tacrine-melatonin Hybrids

Melatonin is a powerful antioxidant natural product that has been used in a number of drug discovery projects to build new molecules for the potential treatment of neurodegenerative diseases.^[45] With this idea in mind, new melatonin-tacrine hybrids have been investigated,^[46] incorporating the hypothesis and concept that “two better than one” regarding the drug design. Thus, the authors have used, in addition to melatonin, typical ferulic or lipoic acid^{[17][47]} as antioxidants, leading to ferulic acid and melatonin tacrine hybrids (**FATMHs**, **VIIa**) (Figure 9), and lipoic acid and melatonin tacrine hybrids (**LATMHs**, **VIIb**) (Figure 9), whose synthesis has carried out in good yields, as a mixture of their non-separable *E/Z* rotamers, using Ugi reaction as the key step (Scheme 2).

As a result of the corresponding extensive biological evaluation, hybrid **FATMH1** (Figure 9) was identified as a very powerful ChEI [IC_{50} (*EeAChE*) = 8.37 ± 0.08 nM; IC_{50} (*eqBuChE*) = 2.91 ± 0.35 nM], strong antioxidant ($9.11 \pm$

Scheme 2. Synthesis of compounds of type **FATMHs** (**VIIa**) and **LATMHs** (**VIIb**).

0.21 TE) and likely to penetrate the BBB *via* passive diffusion. Furthermore, this tacrine presented the best neuroprotection values against the exposed toxic insults (H₂O₂ 300 μ M, A β _{1–40} and A β _{1–42} 30 μ M) at 1 μ M dose and was able to significantly induce the nuclear factor (erythroid-derived 2)-like 2 activators transcription pathway at the concentration of 3 μ M in AREC32 cells, while less hepatotoxic than tacrine.

3.3. Tacrine-neocryptolepine Heterodimers

The bivalent ligand strategy^[40–42] has been used for the synthesis and biological evaluation of tacrine-neocryptolepine heterodimers (**TNC**, **VIII**, Figure 9)^[48] to define potent tacrine analogues, combining in one ligand tacrine and neocryptolepine, an alkaloid isolated from the roots of *Cryptolepis sanguinolenta*,^[49] a plant found in Central and Western Africa. The authors envisioned that the tacrine motif should bind the CAS, while the fully planar aromatic neocryptolepine motif would establish appropriate interactions at the PAS of AChE, resulting in more potent AChEIs than tacrine alone.

As a result of the biological evaluation, **TNC1** (Figure 9) turned out to be a highly potent hChEI, which can be highlighted among the most potent hAChEIs identified so far (IC_{50} hAChE = 0.95 ± 0.04 nM), being also a potent hBuChE inhibitor [IC_{50} (hBuChE) = 2.29 ± 0.14 nM]. On the other hand, ligand **TNC1** presented higher or similar hepatotoxic levels as tacrine. However, considering that the compound was two orders of magnitude more potent than tacrine as AChEI, one would expect the required dose to be lower than in the case of tacrine, which would lead to lower risk of toxicity.

Molecular modelling was carried out using AutoDockVina. To corroborate the flexibility during coupling, flexible torsion was left in the ligand and the dihedral angles rotated freely.

Compound **TNC1** (Figures 10a and 10b) proved to be a dual inhibitor interacting with both CAS (tacrine residues) and PAS (the indoloquinoline unit of neocryptolepine), justifying and supporting the initial hypothesis about the use of a planar aromatic neocryptolepine to reach and bind the PAS, giving these compounds a high inhibitory power on AChE.

ADME-Tox properties were either predicted using the QikProp module from the Schrodinger suite or analyzed with the appropriate assays for molecule **TNC1**, that fulfilled the necessary requirements to be considered a potential CNS drug. The molecule showed values of solubility within limits and

logBB values close to or even greater than 0.3, indicating an excellent potential to traverse the BBB. Regarding the PSA, **TNC1** showed a value around $45 \text{ \AA}^2$, which confirms a good penetration into the CNS, and good oral absorption.

4. Conclusions and Outlook

Since AD is a multifactorial disease, there is an urgent need for designing MSMs able to interact with multiple therapeutic targets involved the pathology. These new molecular entities should be safe, able to easily overpass the BBB, have neuro-protective and antioxidant capabilities and drug-like properties, as well as the ability to interact with other biological targets involved in AD. The studies presented in this review were developed in the framework of the MSM strategy, by designing non-hepatotoxic tacrines, or at least with less hepatotoxic effects than tacrine itself.

Among all the tacrines shown here, it is worth mention that tacrine **IPT** (Figure 6) pertaining to the (benz)imidazolo-pyridinotacrine group (**IV**, Figure 1),^[38] and kojotacrine **KT2** (Figure 7)^[39] were the compounds with the best non-hepatotoxic profile in the *in vitro* cell viability assay that uses the HepG2 cell line, being 6- or 7-fold less hepatotoxic, respectively, than tacrine. Conversely, molecule **TNC1** (Figure 9) of the tacrine-neocryptolepine heterodimer group was the only one presenting hepatotoxic levels higher or similar to tacrine. Despite this unwanted hepatotoxic effects, ligand **TNC1** was the most potent ChEI among all tacrines presented in this review [IC_{50} (hAChE) = $0.95 \pm 0.04 \text{ nM}$; IC_{50} (hBuAChE) = $2.29 \pm 0.14 \text{ nM}$].

Among the tacrine-ferulic acid hybrids group, tacrine **TFAH1** (Figure 9) was the most potent AChEI [IC_{50} (hAChE) = $22.2 \pm 1.6 \text{ nM}$; IC_{50} (hBuChE) = $68.2 \pm 3.9 \text{ nM}$], showing also interesting neuroprotective capacity, which was roughly similar to that tacrine **FATMH1** (Figure 9) of the ferulic acid-tacrine-melatonin hybrid family, a AChEI and BuChEI, 60-fold and 3.4-fold, respectively, less potent than tacrine. Another significant property of molecule **TFAH1** (Figure 9) is its high $A\beta_{1-42}$ inhibition percentage in the ThT assay (65.6%).

Regarding the antioxidant activity, measured with the ORAC test, most of the tacrines shown here presented good antioxidant activity respect to controls. Tacrine **FATMH1** (Figure 9) showed the highest antioxidant power ($9.11 \pm 0.21 \text{ TE}$), although molecule **TFAH1** (Figure 9) was also very potent ($4.29 \pm 0.19 \text{ TE}$).

Last but not least, the *in silico* ADME-Tox properties of the tacrines reviewed here were in line with the parameters required by the Lipinski's rule for drugs to be used for CNS therapies. Thus, most of the tacrines overpassed the BBB. Accordingly, the experimental BBB penetration estimation,

Figure 10. a) Docking pose of compound **TNC1** into hAChE. Ligand is rendered as ball and sticks (carbon atoms in dark green). b) Schematic representation of different interactions of compound **TNC1** with hAChE.

measured in a PAMPA assay, showed that tacrines **QPT4** (Figure 3) (Pe: $4.4 \pm 0.9 \cdot 10^{-6} \text{ cm s}^{-1}$), **TFAH1** (Figure 9) of the TFAHs group (Pe: $61.5 \pm 4.5 \cdot 10^{-6} \text{ cm s}^{-1}$) and **FATMH1** (Figure 9) (Pe: $4.4 \pm 0.8 \cdot 10^{-6} \text{ cm s}^{-1}$), are permeable molecules. For the rest of tacrines, the BBB overpassing capacity was measured *in silico*, taking into account several parameters that could be an indication of their capacity to pass this barrier, such as PSA and QPLogBBB. **QPT4** (Figure 3) had a PSA of $100 \text{ \AA}^2$ for each enantiomer and compound **KT2** (Figure 7) had a PSA of $107 \text{ \AA}^2$ for each enantiomer, much higher than the required value of less than $60 \text{ \AA}^2$ required for drugs acting in the CNS.

The pharmacological properties of the tacrines presented in this review pave the way for designing tacrine analogues with higher inhibition potency against ChEs, better neuroprotective and antioxidant power, able to permeate the BBB and with superior pharmacokinetic properties.

To sum up, we are convinced that tacrine is still a reference for designing new, simple and easily available drugs for AD therapy, showing no hepatotoxic effects, and being able to modulate diverse biological targets involved in the progress and evolution of AD. For this reason, multitarget small tacrines are obviously the type of ligands to be considered for any future perspective on this arena. In particular, the synthesis of tacrines resulting from the juxtaposition with other standards, such as antioxidant ferulic or lipoic acid, and melatonin, or natural products, such as neocryptolepine, as it has been shown here, offer clear opportunities for the identification of new molecules showing improved biological properties respect to the reference ligands for the potential therapy of AD.

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