Synthesis and functional survey of new Tacrine analogs modified with nitroxides or their precursors

Tamás Kálaia, Robin Altmanb, Izumi Maezawac, Mária Balogad, Christophe Morisseau, Jitka Petrolob, Bruce D. Hammock, Lee-Way Jin, James R. Trudell, John C. Voss, Kálmán Hideg

a Institute of Organic and Medicinal Chemistry, University of Pécs, H-7624 Pécs, Szigeti St. 12. Pécs, Hungary
b Department of Biochemistry & Molecular Medicine, University of California Davis, Davis, CA 95616, USA
c M.I.N.D. Institute and Department of Pathology and Laboratory Medicine, University of California Davis, Sacramento, CA95817, USA
d Department of Entomology and UC Davis Comprehensive Cancer Center, University of California Davis, Davis, CA 95616, USA
e Department of Anesthesia, Beckman Program for Molecular and Genetic Medicine, Stanford University, Stanford, CA 94305-5117, USA

A series of new Tacrine analogs modified with nitroxides or pre-nitroxides on 9-amino group via methylene or piperazine spacers were synthesized; the nitroxide or its precursors were incorporated into the Tacrine scaffold. The new compounds were tested for their hydroxyl radical and peroxyl radical scavenging ability, acetylcholinesterase inhibitor activity and protection against Aβ-induced cytotoxicity. Based on these assays, we conclude that Tacrine analogs connected to five and six-membered nitroxides via piperazine spacers (9b, 9b/HCl and 12) exhibited the best activity, providing direction for further development of additional candidates with dual functionality (anti Alzheimer's and antioxidant).

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1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative process that gradually worsens over time. Many clinical symptoms are associated with AD including memory loss, disorientation, language impairment, etc. The etiology of AD has not been elucidated yet, several factors such as amyloid-β (Aβ) deposits [1], τ–protein aggregation, oxidative stress [2], and decreased acetylcholine levels [3] play significant role in the pathology of disease [4]. Currently no treatment is available to cure AD and clinical treatments have only palliative effects. Such treatments include acetylcholinesterase inhibitors (AChEIs) (Tacrine, donepezil, rivastigmine, galantamine) restoring cholinergic deficit, and the NMDA receptor antagonist (memantine) limiting glutamate excitotoxicity. In spite of research efforts, drugs that can reverse or halt the pathology of AD are still lacking. Because of the complexity of AD, a new approach has been proposed for addressing the disease: simultaneous targeting of the multiple pathological processes involved in the neurodegenerative cascade [5]. The approach involves the combination of therapeutic agents that act independently on different etiological targets. This strategy has proven to be successful in treatment of similarly complex diseases such as HIV, cancer and hypertension. In the case of AD, a combination of AChEIs with compounds targeting other pathogenic factors might offer several benefits, at least in the improvement of clinical symptoms of this disease. Several innovative strategies have been published recently, which produced enhanced therapeutic effects over AChEI monotherapy [6].

In the light of this, Tacrine, an AChEI, has been combined with carvedilol [7], melatonin [8], and ferulic acid derivatives [9]. We have recently published the structure of spin-labeled fluorene (SLF), containing a pyrrolidine nitroxide group that provides both increased cell protection against toxicity of Aβ oligomers (AβO) and a route to directly observe the binding of the fluorene to the AβO assembly by EPR spectroscopy [10]. Among the fluorene derivatives, the pyrrolidine nitroxide ring-containing derivatives were found especially useful in counteracting Aβ peptide toxicity, as they possess both antioxidant properties and the ability to disrupt AβO species [10].
Nitroxides are stable free radicals that rapidly cross cell-membranes, preempt free-radical formation by oxidizing redox-active metal ions (equation (1), Fig. 1), and function both as intra- and extracellular SOD mimics (equations (2) and (3)). The reduced form of the nitroxide, hydroxylamine, also has antioxidant activity; a proton and electron donor species (equation (4)). The sterically hindered amine (pre-nitroxide) with ROS scavenging offers a non-toxic stable nitroxide free radical (equation (5)). Several studies indicate that modification of cardioprotective agents [11], PARP-inhibitors [12], or neuroprotective ebselen [13] with nitroxides has a beneficial influence on their activity, being supplemented by the nitroxide’s “in status nascent acting” antioxidants and radical scavengers.

With these concepts in mind, we focused our attention to combine nitroxide and/or nitroxide precursors with 9-amino-1,2,3,4-tetrahydroacridine (Tacrine 1b) in the hope that nitroxides or their precursors (amines and hydroxylamines) may emerge as building blocks in the search of new dual active compounds to confront AD, a disease where oxidative stress contributes significantly to the pathogenesis. In this paper, we report the synthesis and biological study of these Tacrine-nitroxide hybrids by modifying the amino group of Tacrine or by incorporating the nitroxide moiety into the Tacrine scaffold via a substitution of the saturated ring (Scheme 1).

2. Results and discussion

2.1. Chemistry

In order to alkylate the amino-group of Tacrine (1b) [14] it was condensed with aldehydes 2 [15] and 3 [16] in toluene in the presence of piperidine to result in a Schiff-base, which was reduced with lithium aluminum hydride in THF to offer compounds 4b and 5 (Scheme 1). Treatment of 9-chloro-1,2,3,4-tetrahydroacridine 6 [17] with five equivalents of piperazine in refluxing pentanol offered compound 7, a key intermediate, which could be alkylated easily on secondary amine with a paramagnetic allylic bromide 8 [18] to offer compound 9b. Alkylation of 7 with propargyl bromide gave compound 10 which was conjugated with the paramagnetic azide 11 [19] in Cu(1) catalyzed 1,3-dipolar cycloaddition reaction in DMSO [20] to yield 12, the triazol spacer containing compound. Alkylation of compound 7 with 2,3,4-trimethoxybenzylchloride afforded compound 13, which combines an anti-ischemic metabolic agent, trimetazidine [21] and the AChEI Tacrine (Scheme 2).

Reaction of 14 (2-aminobenzonitrile) with 15 (triacetonamine) in the presence of 2.5 equiv aluminum-chloride in dichloroethane [14,22] at reflux temperature after work-up gave compound 16b which could be oxidized with H2O2 in the presence of Na2WO4 to yield nitroxide 17b. Reaction of 4-oxo-TEMPO with 2-aminobenzonitrile in the presence of Lewis acid, despite the consumption of starting materials, did not give compound 17b. For the synthesis of further Tacrine analogs 19b, 20b, 22b and 24b several diamagnetic ketones 18 [23], 21, 23 were incorporated into the Tacrine scaffold under the same conditions used in the synthesis of compound 16b. From these compounds 19b could be oxidized to 20b nitroxide with H2O2 in the presence of Na2WO4, (Scheme 3) To achieve better solubility both amines and nitroxides were converted to HCl salts by treatment with HCl saturated ethanol [24] indicated as 1/HCl, 4/HCl, 9/HCl, 16/HCl, 17/HCl, 20/HCl, 22/HCl, 24/HCl In the case of nitroxides, this resulted in hydroxylamine formation.

Fig. 1. Possible radical scavenging mechanisms and transformations of nitroxides and pre-nitroxides.
2.2. Cholinesterase inhibitory activity and Aβ-induced cytotoxicity protection

In designing new Tacrine derivatives, nitrooxide as an antioxidant building block was bound to amino functional group with the short methylene spacers as for compounds 4b, 9HCl and 12. To demonstrate the importance of the nitrooxide moiety or its precursor diamagnetic compounds 7, 10, 13 also were tested. The other possibility was the incorporation the pyrroline- or tetrahydropyridine nitrooxide ring into the Tacrine scaffold. We synthesized compounds 16b, 17b, 19b, 20b, 22b and 24b with modified Friedlander synthesis. The new Tacrine analogs were tested on hydroxyl radical scavenging activity, peroxyl radical scavenging activity, acetylcholinesterase (from bovine erythrocyte) inhibitory activity, and Aβ-induced cell death inhibitory activity. The ROS scavenging activity and AChEI activity correlation with Aβ-induced cytotoxicity was investigated to find compounds with best antioxidant and anti Alzheimer's activity. As in AD, the memory dysfunction is a consequence of the cholinergic disturbances in the afflicted areas, AChE inhibitors are used to limit the amount of acetylcholine in the brain. In consequence, those cells which are still alive and produce acetylcholine may restore the cholinergic deficit at synaptic sites. For testing Tacrine analogs it was essential to determine AChE inhibitory activity of the new derivatives. Tacrine (1b) and its hydrochloride salt (1HCl) have only acetylcholinesterase activity and do not exhibit any protection against Aβ-induced cytotoxicity. Compounds 4b, 9HCl, 5, 9b, 9HCl, 10 and 12 exhibit less AChEI activity than compound 1b, but their protective concentrations against Aβ-induced cytotoxicity are below 20 μM. While compounds 13, 16b, 16/HCl, 17b, 17/HCl, 19b, 19/HCl, 20b, 20/HCl, 22b, 22/HCl, 24b and 24/HCl also exhibit notable protective activity against the induced cytotoxicity, their AChEI activity is practically lost (over 40 μM), only compounds 24 and 24/HCl produced limited AChEI activity. Hence it can be concluded that beyond compound 13, all the other ineffective analogs contain heterocyclic (pyrroline or tetrahydropyridine) ring incorporated into the Tacrine scaffold, e.g., condensed with a quinoline moiety (Fig. 2.). To understand the difference in AChEI activity of compounds 12, 13 and 16b docking studies were performed.

2.3. Docking of Tacrine derivatives to acetylcholinesterase

A goal of the docking studies was to understand why compound 16b is inactive while compound 12 is active in experimental studies. Fig. 3a and b show a zoomed-in view of the ligands docked into the binding site of acetylcholinesterase. We used a module of Discovery studio 3.5 (DS3.5, Accelrys Inc., San Diego, CA) to search for inter-molecular H-bonds as well as π-bonds. Fig. 3a shows that 16b fits nicely into the binding site, but generates only one π-bond from N25 to Tyr334 of 2CKM. In contrast, compound 12 provided one H-bond between Tyr121 on N25. Moreover, it also made four π-bonds; one from the ring containing C4 to Trp279, one from N36 to Phe330, and two from N36 to each ring of Trp84. When the total energies after the final minimization were compared, compound 16b was −28716.9 versus compound 12 of −28842.9 kcal/mol; a difference of 126 kcal/mol. It is likely that the large difference in
interaction energies between compound 12 and 16b accounts for the lack of activity of compound 16b. The latter molecule provided only one π-bond to the receptor whereas compound 12 provided one H-bond and four π-bonds. We did not calculate the more distributed van der Waals interactions, but they likely also added to the increased interaction energy of the larger compound 12. Docking experiments showed that both Tacrine (1b) and compound 13 are bound equally well and the differences in efficacy at the binding site were not apparent. However, compound 13 structurally more similar to ditacrine (two rigid 1,2,3,4-tetrahydroacridine unit connected with a heptan-1,7-diamine flexible spacer) and the binding energy of compound 13 is somehow less than that of ditacrine (see table in the supplementary information).

2.4. ROS scavenging activity

To study the ROS scavenging activity of the Tacrine analogs, the signal intensity of the BMPO spin trap [25] was measured by EPR spectroscopy following induction of either hydroxyl or superoxide radicals. BMPO produces a distinct EPR line shape, depending on whether the adduct is formed with the hydroxyl or superoxide radical (Fig. 4). Fig. 4 illustrates how Tacrine derivatives with appreciable scavenging activity compete for ROS species (e.g., compound 13), thereby producing a reduced BMPO EPR line intensity. This compares to compound 1b, which has no significant effect on the level of radical detected (Fig. 4). In comparison of the hydroxyl radical scavenging activity and the protective effect against Aβ-induced cytotoxicity, the results are more or less parallel suggesting that free radical processes have a contribution in the induced cytotoxicity. Compounds without nitroxide or nitroxide precursors (1b, 1/HCl, 7, 10) have limited -OH and superoxide scavenging activity, while compounds 4b, 4/HCl 5, 9b, 9/HCl, 12 provide remarkable ROS scavenging activity and protection against Aβ-induced cytotoxicity (Figs. 2 and 5). The exception is the diamagnetic trimetazidine derivative (13), a compound with notable antioxidant activity [26]. Regarding the anellated derivatives, sterically hindered amines (16b, 16/HCl, 19b, 19/HCl)
provide a lower protective activity compared to nitroxides 17b and 20b and their hydroxylamine salt (17/HCl and 20/HCl). Furthermore, the annulated six membered nitroxide (17b) provides superior \( \cdot \)OH scavenging activity compared to the five-membered annulated nitroxide (20b). While several factors may influence the thermodynamics and kinetics of these reactions, a general determining factor is the flexibility of the nitrogen center to planarize upon oxidation or pyramidalize upon reduction [27]. Thus, monocyclic nitroxides (4b, 5, 9b, 12) readily take part in redox processes, as does the six-membered nitroxide with the tetrahydroisoquinoline scaffold (17b). However, in the case of the five-membered rings condensed into an aromatic system (19, 20), the severely constrained ring impairs reactivity at the nitrogen.

As expected, diamagnetic derivatives (1b, 7, 10, 13, 16b, 16/HCl, 20b, 20/HCl, 22b, 22/HCl 24b, 24/HCl) lack peroxyl radical scavenging activity, while the nitroxide or hydroxylamine containing compounds (4b, 4HCl 5, 9b, 9HCl, 12, 17b, 17HCl, 20, 20 HCl) are excellent in this regard. We can also note that paramagnetic Tacrines produce better performance in peroxide scavenging than in HO\(^{\cdot}\) scavenging (Fig. 5). It is well known that Aβ toxicity is related to its redox properties [28] and contributes to oxidative damage by inducing lipid peroxidation, which in turn generates additional cytotoxic free radical formation, leading to mitochondrial and cytoskeletal compromise, depletion of ATP, and ultimate apoptosis. Our findings that nitroxide or its precursor containing Tacrine analogs (4b, 4/HCl 5, 9, 12, 17b, 17/HCl, 20b, 20/HCl) with free radical scavenging capabilities (Fig. 1) corroborate the findings that compounds with antioxidant activity (folate, vitamin E, acetyl-L-carnitine, ferulic acid ethyl ester) provide protection against Aβ--induced toxicity [29,30].

3. Conclusions

A new series of tacrine-nitroxide and nitroxide precursor hybrid related derivatives were synthesized as dual acetylcholinesterase inhibitors and antioxidants (radical scavengers). Our synthesis varied the nitroxide position, tethered to a 9-amino group or anellated to the Tacrine pyridine ring. The influence of the nitroxide ring size was also explored. Compounds were tested on hydroxyl radical scavenging, peroxyl radical scavenging, Aβ--induced cell death and AChE inhibitory assays. The general correlation between ROS scavenging capability and protection against Aβ toxicity is illustrated in Fig. 6. The correlation with hydroxyl scavenging appears a little stronger. It should be noted the solvent and potential carrier components are significantly different in the cell protection assay (e.g., the presence of lipids and proteins). Thus differences in compound solubility (in all assays) or uptake (in the cell protection assay) is likely to produce divergence between the measurements. Such factors are apparent for the dissimilar performance of the base and acid species. Compounds 9 and 12 were the most efficient AChE inhibitors and radical scavengers and exhibited remarkable cell protection toward Aβ--induced toxicity, although compounds 4, 5, 17 and 20 also exhibited notable activity (Fig. 6). This dual protective (AChE inhibitory and antioxidant) profile of compounds 9 and 12 makes these compounds promising leads for developing disease modifying drugs for the future treatment of AD.

4. Experimental protocols

4.1. Chemistry

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a Thermoquest Automass Multi and VG TRIO-2 instruments and in the EI mode. \(^1\)H NMR spectra were recorded with Varian UNITYINOVA 400 WB spectrometer and Bruker Avance 3 Ascend 500. Chemical shifts are referenced to Me\(_4\)Si. Measurements were run at 298 K probe temperature in CDC\(_3\) solution. ESR spectra were taken on Miniscope MS 200 in 10\(^{-4}\) M CHCl\(_3\) solution and monoradicals gave triplet line. Flash column chromatography was performed on Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially available plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF\(_{254}\). Triacetonamine, 1,2,2,6,6-pentamethylpiperidine, 1-methyl piperidine, compound 14, Tacrine and all other chemicals were purchased from Aldrich, compound 2 [15], 3 [16], 6 [17], 8 [18], 11 [19], 18 [23] were prepared as described earlier.

4.1.1. Synthesis of 9-amino substituted Tacrines

In a 250 mL round bottomed flask equipped with a Dean–Stark constant separator which is connected to a reflux condenser, a solution of Tacrine (1) (1.98 g, 10 mmol) and aldehyde 2 or 3 (10.0 mmol) and piperidine 85 mg (1.0 mmol) in toluene (100 mL) was heated on reflux temperature for 24 h. After cooling the solvent was evaporated off and the residue was purified by flash column chromatography to remove starting materials. To the solution of Schiff-base in dry THF (30 mL) under N\(_2\) at 0 °C LiAlH\(_4\) (20.0 mmol, 8.3 mL) was added dropwise. After consumption of the Schiff-base (~2 h) the mixture was poured on mixture of ice and 10% aq. NaOH.

![Image](image-url)
solution (100 mL) and the mixture was stirred at room temperature for 30 min. The organic phase was separated, and the aqueous phase was extracted with CH₂Cl₂ (2 × 20 mL). The combined organic phase was dried (MgSO₄), filtered and evaporated and the residue was purified by flash column chromatography (hexane–EtOAc, CHCl₃–Et₂O) to yield the title compounds as yellow crystalline solids.

4.1.1.2. 2,2,6,6-Tetramethyl-4-[(1,2,3,4-tetrahydroacridin-9-yl)amino]methyl-2,3,6-tetrahydro-1H-pyrrrol-1-yl oxol radical (5) (HO–4637). 800 mg (22%), mp 157–159 °C. Ms (EI) m/z (%): 364 (M⁺, 14), 334 (278), 211 (100), 197 (45). Anal calcd. for: C₂₃H₃₀N₃O: C 75.95, H 8.05, N 11.86.

4.1.1.3. 9-(4-[(2,3,4-Trimethoxybenzyl)piperazin-1-yl]-1,2,3,4-tetrahydroacridine (13) (HO–4392). 127 mg (55%), yellow solid, mp 202–204 °C. H NMR (2HCl salt in D₂O) δ = 8.14 (d, 1H, J = 8 Hz), 7.90 (s, 2H), 7.73 (t, 1H, J = 8 Hz), 7.28 (d, 1H, J = 8 Hz), 6.95 (d, 1H, J = 8 Hz), 4.46 (s, 2H, J = 8 Hz), 3.88 (s, 3H, 12), 3.52 (m, 2H), 3.21 (s, 2H, J = 6 Hz), 2.90 (t, 2H, J = 6 Hz), 1.95 (m, 2H), 1.86 (m, 2H). 13C NMR (CD₃OD): δ = 161.5, 152.5, 154.0, 148.4, 143.5, 129.8, 129.0, 128.4, 127.2, 126.3, 125.3, 124.2, 108.7, 61.8, 61.2, 57.8, 56.5, 55.1, 51.4, 34.4, 27.8, 23.9, 23.6. Ms (EI) m/z (%): 447 (M⁺, 12), 266 (90), 238 (44), 181 (100). Anal calcd for: C₂₇H₃₂N₂O: C 72.46, H 7.43, N 9.39; found: C 72.45, H 7.46, N 9.41.

4.1.4. Modification of the Taurine analog by a nitrosite with click-reaction. 2,2,6,6-Tetramethyl-4-[(1,2,3,4-tetrahydroacridin-9-yl)amino]methyl-1,1,3,3-tetramethyl-1,2,3,4-tetrahydrobenzo[1,6]naphthyridines and 1H-pyrrolo[3,4-b]quinoline general procedure (16b, 19b, 22b, 24b).

Mixture of cyclic ketone 15 or 18 or 21 or 23 (10.0 mmol) and 2-aminobenzonitrile (14) (118 g, 10.0 mmol) in 1,2-dichloroethane (30 mL) was stirred at room temperature for 10 min, then anhydrous AlCl₃ (3.32 g 25.0 mmol) was added in one portion and the mixture was stirred and refluxed for 2 h. After cooling the mixture was basified with 10% aq. NaOH (100 mL), the mixture was stirred at ambient temperature for 30 min. After separation of organic phase the aqueous phase was extracted with CH₂Cl₂ (2 × 20 mL). The combined organic phase was dried (MgSO₄), filtered and evaporated. The residue was purified by flash column chromatography (hexane–EtOAc or CHCl₃–Et₂O) to give the title compounds as solids in 32–52% yield.

4.1.5. Friedlander reaction for synthesis of 1,2,3,4-tetrahydrobenzof[b][1,6]naphthyridines and 1H-pyrrolo[3,4-b]quinoline general procedure (16b, 19b, 22b, 24b). A stirred solution of compound 7 (1.33 g, 5.0 mmol) and compound 8 (5.5 mmol) or propargyl bromide or 2,3,4-trimethoxybenzyl chloride (5.5 mmol) and K₂CO₃ (760 mg, 5.5 mmol) in CHCl₃ (30 mL) was heated at reflux till the consumption of starting material (3 h). After cooling the inorganic salt was filtered off, the organic phase was washed with water (10 mL) and extracted with CHCl₃ (2 × 20 mL). The organic phase was dried (MgSO₄), filtered and evaporated. The residue was purified by flash column chromatography (CHCl₃–Et₂O) to furnish the title compounds.

4.1.3. General procedure for alklylation of 9-piperazin-1-yl-1,2,3,4-tetrahydroacridines (9b, 10, 13). A stirred solution of compound 7 (1.33 g, 5.0 mmol) and compound 8 (5.5 mmol) or propargyl bromide or 2,3,4-trimethoxybenzyl chloride (5.5 mmol) and K₂CO₃ (760 mg, 5.5 mmol) in CHCl₃ (30 mL) was heated at reflux till the consumption of starting material (3 h). After cooling the inorganic salt was filtered off, the organic phase was washed with water (10 mL), the organic phase was separated, dried (MgSO₄), filtered and evaporated. The residue was purified by flash column chromatography (CHCl₃–Et₂O) to furnish the title compounds.

4.1.3.1. 2,2,5,5-Tetramethyl-3-[(2,3,4,5-tetrahydroacridin-9-yl) piperazin-1-ylmethyl]-2,5-diiodo-1H-pyrrrol-1-yl oxol radical (9b) (HO–4380). 130 mg (62%), yellow solid, mp: 138–140 °C. Ms (EI) m/z (%): 419 (M⁺, 43), 389 (S), 280 (75), 267 (82), 225 (100). Anal calcd. for: C₂₉H₂₃N₃O: C 74.43; H 8.41; N 13.35. Found: C 74.29, H 8.33, N 13.26.

4.1.3.2. 9-[(Prop-2-yn-1-yl)-1H-pyrrol-1-yl-1,2,3,4 tetrahydroacridine (10) (HO–4563). 119 mg (78%) yellow solid, mp 94–96 °C. H NMR (CD₃OD) δ = 8.17 (d, 1H, J = 9 Hz), 7.85 (d, 1H, J = 8 Hz), 7.60 (t, 1H, J = 7 Hz), 7.46 (t, 1H, J = 8 Hz), 3.44 (d, 2H, J = 2 Hz), 3.37 (t, 4H, J = 5 Hz), 3.06 (t, 2H, J = 7 Hz), 2.97 (t, 2H, J = 6 Hz), 2.81 (t, 3H, J = 5 Hz), 2.75 (s, 1H), 1.96 (m, 2H), 1.86 (m, 2H). 13C NMR (CD₃OD) δ = 161.5, 155.1, 148.4, 129.9, 129.2, 128.4, 127.1, 126.4, 125.2, 79.1, 75.4, 53.9, 51.1, 47.8, 34.4, 27.8, 23.9, 23.6. Ms (EI) m/z (%): 305 (M⁺, 38), 266 (11), 209 (48), 67 (100). Anal calcd. for: C₂₀H₂₃N₂: C 78.65; H 7.50; N 13.76; found: C 78.55, H 7.51, N 13.57.
4.1.6.1. 10-Amino-1,1,3,3-tetramethyl-3,4-dihydrobenzo[b][1,6]naphthoquinone

Random ligand conformations are generated using high-dynamics (MD) scheme to dock ligands into a receptor binding site. The CHARMm force field is closer in size and length to the planned ligands; compound 12b docked (PDB ID 2CKM). We chose the latter structure as di-tacrine receptor with Tacrine docked (PDB ID 1ACJ) and one with di-tacrine (HO4277). We considered two X-ray structures also formed) appears (12b) (HO4330). Anal calcd for: C15H18N3O: C 75.62, H 8.52, N 15.76.

4.1.6.2. 9-Amino-1,1,3,3-tetramethyl-1H-pyrrolo[3,4-b]quinolinderivative (12b) (HO4330). 1H NMR (CDCl3) δ = 7.96 (d, 1H, J = 8 Hz), 7.51 (t, 1H, J = 8 Hz), 7.29 (1H, t, J = 8 Hz) 2.97 (s, 2H), 2.54 (s, 3H), 1.76 (s, 6H), 1.17 (s, 6H).13C NMR of 2 HCl salt δ: 154.7, 145.6, 137.4, 134.5, 129.9, 122.9, 119.1, 114.9, 101.0, 49.6, 49.2, 43.1, 24.4. Ms (EI) m/z (%): 269 (M+), 54 (24%), 223 (39%). Anal calcd for: C15H18N3: C 75.80; H 8.61; N 15.60; found: C 75.62, H 8.52, N 15.76.

4.1.6. Oxidation of compounds 16b and 19b–17b and 20b nitrooxide free radicals

To a stirred solution of amine 16b or 19b (5.0 mmol) and Na2WO4·2H2O (164 mg, 0.5 mmol) in EtOH (20 mL) and water 5 (5 mL) 30% aq. H2O2 (5 mL) was added dropwise at 0 °C. After the addition the stirring was continued at room temperature and the course of the reaction was followed by TLC (hexane/EtOAc 2:1) and once by product (upon prolonged oxidation time pyridine-N-oxyl also formed) appears (~10 h) the organic solvent was evaporated and the mixture was extracted with CHCl3 (2 × 30 mL). The organic phase was dried (MgSO4) filtered and evaporated to offer the title nitroxides 17b or 20b as yellow solids.

4.1.6.1. 10-Amino-1,1,3,3-tetramethyl-3,4-dihydrobenzo[b][1,6]naphthoquinone-10-amine (24b) (HO4330). 1H NMR (CDCl3) δ = 7.96 (d, 1H, J = 8 Hz), 7.51 (t, 1H, J = 8 Hz), 7.39 (d, 1H, J = 8 Hz), 7.29 (1H, t, J = 8 Hz) 2.97 (s, 2H), 2.54 (s, 3H), 1.76 (s, 6H), 1.17 (s, 6H).13C NMR of 2 HCl salt δ: 154.7, 145.6, 137.4, 134.5, 129.9, 122.9, 119.1, 114.9, 101.0, 49.6, 49.2, 43.1, 24.4. Ms (EI) m/z (%): 269 (M+), 54 (24%), 223 (39%). Anal calcd for: C15H18N3: C 75.80; H 8.61; N 15.60; found: C 75.62, H 8.52, N 15.76.

4.1.6.2. 9-Amino-1,1,3,3-tetramethyl-1H-pyrrolo[3,4-b]quinolinoderivative (20b) (HO4279). 281 mg (22%), yellow solid, mp > 230 °C. Ms (EI) m/z (%): 256 (M+, 62), 241 (45), 226 (77), 211 (100). Anal calcd for: C15H18N3O: C 71.08, H 7.46, N 15.54 found: C 71.96; H 7.56; N 15.46.

4.2. Molecular modeling of compounds 12b and 16b

The structures of Tacrine, di-tacrine, compound 12b, and compound 16b were obtained. They were prepared for docking with the Generate Conformations module of Discovery Studio 3.5 (DS3.5, Accelrys Inc, San Diego, CA, USA). We considered two X-ray structures of acetylcholinesterase as the receptor for docking studies; a receptor with Tacrine docked (PDB ID 1TAC) and one with di-tacrine docked (PDB ID 2CKM). We chose the latter structure as di-tacrine is closer in size and length to the planned ligands; compound 16b (inactive in our assays) and compound 12b (active in our assays). The acetylcholinesterase structure 2CKM was prepared for docking by adding hydrogens, checking for missing atoms, and assigning the CHARMM force field to all atoms. We used the CDOCKER module of DS3.5. CDOCKER uses a CHARMM-based molecular dynamics (MD) scheme to dock ligands into a receptor binding site. Random ligand conformations are generated using high-energy MD. The conformations are then translated into the binding site. Candidate poses are then created using random rigid-body rotations followed by simulated annealing. A final minimization with the CHARMM force field is then used to refine the ligand poses. We highlighted di-tacrine in the crystal structure of 2CKM and defined a sphere that contained it; approximately 11 A. This sphere limited the possible positions of the ligands in subsequent docking runs. We used 1000 steps of molecular dynamics at 1000 K to produce ligand conformations for docking. After docking, we selected the ten top ‘Hits’ for refinement using molecular dynamics with simulated annealing. For each selected pose, we used 2000 steps of heating to a target temperature of 700 K and then 5000 steps of cooling to a target temperature of 300 K. Then we optimized each of the resulting poses to a gradient of 0.001 kcal/mol with DS3.5. We ranked the poses based on two criteria; CDOCKER energy and CDOCKER interaction energy. The latter value was the most informative, it is the difference between the total final energy of the docked complex and the sum of the energies of the receptor and the ligand. We chose the best complex of compound 16b and compound 12b based on the CDOCKER interaction energy value. For these two structures we performed an optimization of the entire receptor plus each ligand with a light restraining force of 10 kcal/(mol × A).
4.2.3. Acetylcholinesterase inhibition assay

The acetylcholinesterase inhibitory activity of the tacrine was determined using acetyl-thio-choline and DTNB according to the method previously published by Wilson and Henderson [33]. Acetylcholinesterase from bovine erythrocytes was used in the assay at a final concentration of 80 μg/mL, and the buffer used for the measurements was 100 mM NaPO₄ (pH 8.0) containing 0.1 mg/mL BSA. Results are expressed as means ± standard deviation of at least three separate experiments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.026.

References