Rational Approach to Discover Multipotent anti-Alzheimer Drugs

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Supporting Information

Contents of SI: Contains experimental details for the synthesis and for the determination of the biological activity, spectra data and elemental analysis data for all new compounds.

1. Synthesis and characterization of compounds 1-8

Compounds **1-8** were synthesized according to Scheme 1, coupling tetrahydroacridine intermediates with lipoic acid.

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR, electron impact (EI) mass, and direct infusion ESI-MS spectra were recorded on Perkin-Elmer 297, VG 7070E, and Waters ZQ 4000 apparatus, respectively. ^{1}H NMR, ^{13}C NMR, gHSQC and COSY experiments were recorded on Mercury 400 and Varian VXR 200 and 300 instruments. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), or m (multiplet). Although the IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. The elemental compositions of the compounds agreed to within ± 0.4% of the calculated value. When the elemental analysis is not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040-0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063-0.200 mm; Merck) chromatography. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry.

(3-Aminomethyl-6-chloro-1,2,3,4-tetrahydroacridin-9-yl)amine (16). The synthesis of compound **16** was achieved by condensation of 2-amino-4-chlorobenzonitrile with 3-nitromethylcyclohexanone followed by reduction of the nitro group according to Rosini et al.,[1] and the isomeric conformation was assigned by means of ^{1}H NMR, ^{13}C NMR, gHSQC, and COSY experiments. Total yield 30%; mp 285-288 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 7.91 (d, J = 8.9 Hz, 1H, C8-H), 7.58 (d, J = 2.3 Hz, 1H, C5-H), 7.19 (dd, J = 9.0, 2.3 Hz, 1H, C7-H), 2.86-2.94 (m, 1H, C4-H), 2.60-2.69 (m, 3H, -CH_2NH$_2$, C1-H), 2.19-2.25 (m, 2H, C1-H, C4-H), 2.04-2.13 (m, 1H, C2-

H), 1.75-1.83 (m, 1H, C3-H), 1.29-1.39 (m, 1H, C2-H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.9, 150.3, 147.7, 135.2, 126.1 (C5), 124.6 (C7), 124.1 (C8), 116.3, 110.4, 48.1 (-*C*H_2NH_2), 38.3 (C4), 37.9 (C3), 27.3 (C2), 24.2 (C1); EI MS *m/z* 261 (M^+).

General procedure for the synthesis of compounds 1-8.

A solution of the appropriate tetrahydroacridinamine (1 eq) and lipoic acid (1.5 eq) in dry DMF (5 mL), under N_2, was cooled to 0 °C and then treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (1.2 eq): the mixture was stirred at 0 °C for further 15 min and then at rt for 2 h in the dark. Solvent was then removed under vacuum, avoiding heating up the reaction mixture, affording an oily residue that was purified by gravity column.

5-([1,2]Dithiolan-3-yl)-*N*-{[2-(1,2,3,4-tetrahydroacridin-9-yl)amino]ethyl}pentanamide (1). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)ethane-1,2-diamine (**9**)[2] (140 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.055) afforded **1** as a foam solid: 35% yield; ^{1}H NMR (300 MHz, CD_3OD) δ 8.12 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* = 8.8 Hz, 1H), 7.58, (t, *J* = 8.2 Hz, 1H), 7.39 (t, *J* = 8.2 Hz, 1H), 3.70 (t, *J* = 6.3 Hz, 2H), 3.28-3.39 (m, 3H), 2.93-3.15 (m, 4H), 2.71-2.79 (m, 2H), 2.26-2.40 (m, 1H), 2.15 (t, *J* = 8.6 Hz, 2H), 1.64-1.93 (m, 5H), 1.30-1.61 (m, 6H); MS (ESI^+) *m/z* 430 $(M+H)^+$. Calcd. for $C_{23}H_{31}N_3OS_2$: C, 64.30; H, 7.27; N, 9.78; found C, 64.41; H, 7.28; N, 9.75.

5-([1,2]Dithiolan-3-yl)-*N*-{[3-(1,2,3,4-tetrahydroacridin-9-yl)amino]propyl}pentanamide (2). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (**10**) (100 mg, obtained from 9-chloro-1,2,3,4-tetrahydroacridine and propane-1,3-diamine following the procedure described in Carlier et al.,[3] and purified by flash chromatography with a step gradient system of CH_2Cl_2/MeOH/ aqueous 30% ammonia (9.5:0.5:0.0 to 7:3:0.1): 65% yield, ^{1}H NMR (200 MHz, CD_3OD) δ 8.08 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* = 8.7 Hz, 1H), 7.53, (t, *J* = 8.3 Hz, 1H), 7.32 (t, *J* = 8.3 Hz, 1H), 3.54 (t, *J* = 6.7 Hz, 2H), 2.87-2.98 (m, 2H), 2.65 (t, *J* = 7.5 Hz, 4H), 1.64-1.93 (m, 6H)). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (5:4:1:0.05) afforded **2** as a foam solid: 35% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.15 (d, *J* = 8.8 Hz, 1H), 7.78 (d, *J* =

8.8 Hz, 1H), 7.56-7.64 (m, 1H), 7.37-7.44 (m, 1H), 3.69 (t, *J* = 6.6 Hz, 2H), 3.40-3.52 (m, 1H), 3.23-3.36 (t, *J* = 6.6 Hz, 2H), 2.92-3.18 (m, 4H), 2.74-2.83 (m, 2H), 2.28-2.43 (m, 1H), 2.19 (t, *J* = 7.1 Hz, 2H), 1.73-1.95 (m, 7H), 1.22-1.68 (m, 6H);); MS (ESI^+) *m/z* 444 $(M+H)^+$. Calcd. for $C_{24}H_{33}N_3OS_2$: C, 64.97; H, 7.50; N, 9.47; found C, 65.18; H, 7.52; N, 9.44.

5-([1,2]Dithiolan-3-yl)-*N*-{[4-(1,2,3,4-tetrahydroacridin-9-yl)amino]butyl}pentanamide (3). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)butane-1,4-diamine (**11**)[3] (290 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.06) afforded **3** as a foam solid: 38% yield; ^1H NMR (200 MHz, CD_3OD) δ 8.12 (d, *J* = 8.6 Hz, 1H), 7.78 (d, *J* = 8.6 Hz, 1H), 7.52-7.62 (m, 1H), 7.32-7.43 (m, 1H), 3.41-3.60 (m, 3H), 2.90-3.21 (m, 6H), 2.68-2.77 (m, 2H), 2.31-2.46 (m, 1H), 2.17 (t, *J* = 6.9 Hz, 2H), 1.38-1.95 (m, 15H); MS (ESI^+) *m/z* 458 $(M+H)^+$. Calcd. for $C_{25}H_{35}N_3OS_2$: C, 65.60; H, 7.71; N, 9.18; found C, 65.67; H, 7.69; N, 9.15.

5-([1,2]Dithiolan-3-yl)-*N*-{[5-(1,2,3,4-tetrahydroacridin-9-yl)amino]pentyl}pentanamide (4). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)pentane-1,5-diamine (**12**)[3] (480 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.055) afforded **4** as a foam solid: 40% yield; ^1H NMR (200 MHz, CD_3OD) δ 8.09 (d, *J* = 8.6 Hz, 1H), 7.78 (d, *J* = 8.6 Hz, 1H), 7.52-7.60 (m, 1H), 7.33-7.41 (m, 1H), 3.40-3.57 (m, 3H), 2.87-3.18 (m, 6H), 2.63-2.75 (m, 2H), 2.25-2.43 (m, 1H), 2.17 (t, *J* = 6.8 Hz, 2H), 1.35-1.95 (m, 17H); MS (ESI^+) *m/z* 472 $(M+H)^+$. Calcd. for $C_{26}H_{37}N_3OS_2$: C, 66.20; H, 7.91; N, 8.91; found C, 66.41; H, 7.89; N, 8.88.

5-([1,2]Dithiolan-3-y)-N-{[6-(1,2,3,4-tetrahydroacridin-9-yl)amino]hexyl}pentanamide (5). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)hexane-1,6-diamine (**13**)[3] (370 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.05) afforded **5** as a foam solid: 30% yield; ^1H NMR (200 MHz, $CDCl_3$) δ 7.83 (apparent t, *J* = 9.3 Hz, 2H), 7.47-7.56 (m, 1H), 7.28-7.37 (m, 1H), 5.89 (t, *J* = 3.2 Hz, 1H, exchangeable with D_2O), 4.15 (br s, 2H, exchangeable with D_2O), 3.40-3.57 (m, 3H), 3.01-3.23 (m, 6H), 2.60-2.75 (m, 2H), 2.31-2.48 (m, 1H), 2.15 (t, *J* = 7.3 Hz, 2H), 1.35-1.96 (m, 19H); MS (ESI^+) *m/z* 486 $(M+H)^+$. Calcd. for $C_{27}H_{39}N_3OS_2$: C, 66.76; H, 8.09; N, 8.65; C, 66.87; H, 8.12; N, 8.62.

5-([1,2]Dithiolan-3-y)-*N*-{[7-(1,2,3,4-tetrahydroacridin-9-yl)amino]heptyl}pentanamide (6). It was synthesized from N^1-(1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine (**14**)[3] (220 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.05) afforded **6** as a foam solid: 35% yield; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.92 (apparent t, *J* = 9.4 Hz, 2H), 7.51-7.61 (m, 1H), 7.30-7.41 (m, 1H), 5.57 (t, *J* = 3.2 Hz, 1H, exchangeable with D_2O), 3.40-3.61 (m, 3H), 3.01-3.24 (m, 6H), 2.64-2.73 (m, 2H), 2.38-2.54 (m, 1H), 2.18 (t, *J* = 7.3 Hz, 2H), 1.25-1.98 (m, 21H); MS (ESI$^+$) *m/z* 500 (M+H)$^+$. Calcd. for $C_{28}H_{41}N_3OS_2$: C, 67.29; H, 8.27; N, 8.41; C, 67.43; H, 8.30; N, 8.39.

5-([1,2]Dithiolan-3-yl)-*N*-[3-(6-chloro-1,2,3,4-tetrahydro-acridin-9-yl)amino]propyl}pentanamide (7). It was synthesized from *N*1-(6-chloro-1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (**15**) (180 mg, obtained from 6,9-dichloro-1,2,3,4-tetrahydroacridine and propane-1,3-diamine following the procedure described in Carlier et al.,[3] and purified by flash chromatography with a step gradient system of CH_2Cl_2/MeOH/ aqueous 30% ammonia (9.5:0.5:0.0 to 8:2:0.03): 70% yield, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.93 (d, *J* = 9.1 Hz, 1H), 7.86 (d, *J* = 2.4 Hz, 1H), 7.22 (dd, *J* = 9.0, 2.3 Hz, 1H), 3.62 (t, *J* = 6.8 Hz, 2H), 2.88-3.05 (m, 4H), 2.60-2.68 (m, 2H), 1.71-1.95 (m, 6H)). Elution with petroleum ether/CH_2Cl_2/EtOH/aqueous 30% ammonia (7:2:1:0.03) afforded **7** as a foam solid: 35% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.08 (d, *J* = 8.9 Hz, 1H), 7.72 (d, *J* = 2.1 Hz, 1H), 7.28 (dd, *J* = 8.9, 2.1 Hz, 1H), 3.42-3.58 (m, 3H), 3.27 (t, *J* = 6.5 Hz, 2H), 2.89-3.17 (m, 4H), 2.65-2.77 (m, 2H), 2.27-2.43 (m, 1H), 2.19 (t, *J* = 7.2 Hz, 2H), 1.73-1.91 (m, 7H), 1.31-1.65 (m, 6H); MS (ESI$^+$) *m/z* 478 (M+H)$^+$. Calcd. for $C_{24}H_{32}ClN_3OS_2$: C, 60.29; H, 6.75; N, 8.79; found C, 60.45; H, 6.74; N, 8.77.

***N*-[(9-Amino-6-chloro-1,2,3,4-tetrahydroacridin-3-yl)methyl]-5-[1,2]dithiolan-3-yl)pentanamide (8)**. It was synthesized from **16** (150 mg). Elution with CH_2Cl_2/toluene/EtOH/aqueous 30% ammonia (5:3:2:0.02) afforded **8** as a foam solid: 30% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.09 (d, *J* = 8.9 Hz, 1H), 7.72 (d, *J* = 2.2 Hz, 1H), 7.36 (dd, *J* = 9.2, 2.2 Hz, 1H), 3.50-3.62 (m, 2H), 2.96-3.21 (m, 4H), 2.70-2.83 (m, 1H), 2.38-2.69 (m, 3H), 2.28 (t,

7.0 Hz, 2H), 2.05-2.21 (m, 2H), 1.79-1.95 (m, 1H), 1.23-1.78 (m, 7H); EI MS *m/z* 449 (M^+). Calcd. for $C_{22}H_{28}ClN_3OS_2$: C, 58.71; H, 6.27; N, 9.34; found C, 58.91; H, 6.26; N, 9.31.

2. Biology

Inhibition of AChE and BChE. The method of Ellman et al. was followed.[4] Five different concentrations of each compound were used in order to obtain inhibition of AChE or BChE activity comprised between 20-80%. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.02 unit/mL of human recombinant AChE or human serum BChE (Sigma Chemical), and 550 μM of substrate (acetylthiocholine iodide or butyrylthiocholine iodide). Test compounds were added to the assay solution and preincubated at 37 °C with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE or BChE in order to account for non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration–inhibition curves.

Determination of Steady State Inhibition Constant. To obtain estimates of the competitive inhibition constant K_i, reciprocal plots of 1/V versus 1/[S] were constructed at relatively low concentration of substrate (below 0.5 mM). The plots were assessed by a weighted least square analysis that assumed the variance of V to be a constant percentage of V for the entire data set. Slopes of these reciprocal plots were then plotted against the concentration of **7** (range 0 – 0.344 nM) in a weighted analysis and K_i was determined as the ratio of the replot intercept to the replot slope.

Reciprocal plots involving TC (not shown) or **7** inhibition show both increasing slopes (decreased V_{max} at increasing inhibitor's concentrations) and increasing intercepts (higher K_m) with higher inhibitor concentration. This pattern indicates mixed inhibition, arising from significant inhibitor interaction with both the free enzyme and the acetylated enzyme. Replots of the slope

versus the concentration of **7** or TC gives estimate of competitive inhibition constant, $K_i = 0.155 \pm 0.046$ nM or $K_i = 0.151 \pm 0.016$ μM, respectively.

So the pattern in the graphical representation shows **7** able to bind to the peripheral anionic site as well as the active site of AChE.

Inhibition of AChE-induced Aβ aggregation. Aliquots of 2 μL Aβ peptide, lyophilized from 2 mg mL^{-1} 1,1,1,3,3,3-hexafluoro-2-propanol solution and dissolved in DMSO, were incubated for 24 h at room temperature in 0.215 M sodium phosphate buffer (pH 8.0) at a final concentration of 230 μM. For co-incubation experiments aliquots (16 μL) of AChE (final concentration 2.30 μM, Aβ/AChE molar ratio 100:1) and AChE in the presence of 2 μL of the tested inhibitor in 0.215 M sodium phosphate buffer pH 8.0 solution (final inhibitor concentration 100 μM) were added.

Blanks containing Aβ, AChE, and Aβ plus inhibitors at various concentrations, in 0.215 M sodium phosphate buffer (pH 8.0) were prepared. The final volume of each vial was 20 μL. Each assay was run in duplicate. To quantify amyloid fibril formation, the thioflavin T (ThT) fluorescence method was then applied.[5] After dilution with glycine-NaOH buffer (pH 8.5), containing 1.5 mM ThT, the fluorescence intensities due to β-sheet conformation was monitored for 300 s at $\lambda_{em} = 490$ nm ($\lambda_{ex} = 446$ nm). The percent inhibition of the AChE induced aggregation due to the presence of the test compound was calculated by the following expression: $100-(IF_i/IF_o \times 100)$ where IF_i and IF_o are the fluorescence intensities obtained for Aβ plus AChE in the presence and in the absence of inhibitor, respectively, minus the fluorescent intensities due to the respective blanks.

Cell cultures. Human neuronal-like cells, SH-SY5Y, were routinely grown at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Determination of cytotoxicity. The cytotoxicity was evaluated with the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay, as described by Mosmann et al.[6] Briefly, SH-SY5Y cells were seeded in 96-well microtiter plates at 2×10^5 cells/well. After

24 h of incubation at 37°C in 5% CO_2, the growth medium was removed and media containing compounds (0.1-50 μM) were added to the cells. After 24 h of incubation, the cells were washed with phosphate buffered saline (PBS) and then incubated with MTT (5 mg/ml) in PBS for 4 h. After removal of MTT and further washing, the formazan crystals were dissolved with isopropanol. The amount of formazan was measured (405 nm) with a spectrophotometer (TECAN⟩ , Spectra model Classic, Salzburg, Austria). The cell viability was expressed as percentage of control cells and calculated by the formula F_t/F_{nt} x 100, where F_t = absorbance of treated neurones and F_{nt} = absorbance of untreated neurones.

Determination of antioxidant activity. The antioxidant activity of compounds was evaluated by measuring the formation of intracellular reactive oxygen species (ROS) evoked by exposure of SH-SY5Y cells to *tert*-butyl hydroperoxide (*t*-BuOOH), a compound used to induce oxidative stress. Formation of intracellular ROS was determined using a fluorescent probe, DCFH-DA, as described by Wang H. et al.[7] Briefly, SH-SY5Y cells were seeded in 96-well microtiter plates at 2 × 10^5 cells/well. After 24 h of incubation at 37°C in 5% CO_2, the growth medium was removed and media containing compounds (0.1-50 μM) were added to the cells. After 24 h of incubation, the cells were washed with PBS and then incubated with 5μM of DCFH-DA in PBS at 37°C in 5% CO_2 for 30 min. After removal of DCFH-DA and further washing, the cells were incubated with 0.1 mM *t*-BuOOH in PBS for 30 min. At the end of incubation, the fluorescence of the cells from each well was measured ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 535 nm) with a spectrofluorometer (Wallac Victor⟩ Multilabel Counter, Perkin Elmer Inc., Boston, MA). The results were expressed as percentage increase of intracellular ROS evoked by exposure to *t*-BuOOH and calculated by the formula [(F_t – F_{nt}) /F_{nt} x 100], where F_t = fluorescence of treated neurones and F_{nt} = fluorescence of untreated neurones.

Statistical analysis. Data are reported as mean ± SD of at least 3 independent experiments. Statistical analysis was performed using ANOVA (Scheffe post hoc test was used) and the

differences were considered significant at $p < 0.05$. Analyses were performed using STATISTICA 4.5 software on a Windows platform.

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