

Supporting Information

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**Specific Targeting of Acetylcholinesterase and Butyrylcholinesterase
Recognition Sites. Rational Design of Novel, Selective and Highly Potent
Cholinesterase Inhibitors.**

Experimental Section.

Melting points were determined using an Electrothermal 8103 apparatus and are uncorrected. IR spectra were taken with Perkin-Elmer 398 and FT 1600 spectrophotometers. ^1H -NMR spectra were recorded on a Bruker 200 MHz spectrometer with TMS as internal standard; the values of the chemical shifts (δ) are given in ppm and coupling constants (J) in Hz. All chemicals used were of reagent grade, and all reactions were carried out under argon atmosphere. Progress of the reaction was monitored by TLC on silica gel plates (Riedel-de-Haen, Art. 37341). Merck silica gel (Kieselgel 60) was used for flash chromatography (230-400 mesh) columns, and a Dowex 50WX8-400 resin was used to purify the final products. Extracts were dried over MgSO_4 , and solvents were removed under reduced pressure. Elemental analyses were performed on a Perkin-Elmer 240C elemental analyzer, and the results are within $\pm 0.4\%$ of the theoretical values. Yields refer to purified products and are not optimized.

Synthesis of compound 2

Ethyl 2-(3,5-dichlorophenylamino)-1-cyclohexencarboxylate. A mixture of 3,5-dichloroaniline (5.00 g, 31 mmol) and ethyl 2-oxocyclohexanecarboxylate (5.79 g, 34 mmol) was heated in a steam bath for 8 h. After cooling the crude solid residue, and purification by crystallization from methanol, the title compound was obtained as a yellow powder. Yield 75%, mp 72-75 °C. ^1H NMR (CDCl_3) δ 1.28, (t, 3H, $J=7.0$ Hz), 1.60-1.63 (m, 4H), 2.34-2.37 (m, 4H), 4.16 (q, 2H, $J=7.0$ Hz), 6.90-7.01 (m, 3H), 10.89 (br s, 1H). Anal. ($\text{C}_{15}\text{H}_{17}\text{Cl}_2\text{NO}_2$) C, H, N.

6,8-Dichloro-1,2,3,4-tetrahydro-9-hydroxyacridine. The intermediate described above (2.00 g, 6.40 mmol) was dissolved in hot diphenylether and the flask was fitted with a Dean-Stark trap to remove EtOH. The mixture was heated at reflux until the starting material disappeared (about 20-30 min.) (TLC, EtOAc/MeOH, 95:5). After cooling to room temperature, hexane (50 mL) was added and the resulting solid, collected by filtration and washed with additional hexane, was recrystallized from EtOH to yield the title compound as a yellow powder. Yield 67%, mp >300 °C. ^1H NMR

(DMSO- d_6) δ 1.65-1.68 (m, 4H), 2.47-2.48 (m, 2H), 2.56-2.62 (m, 2H), 7.21 (d, 1H, $J=1.7$ Hz), 7.39 (d, 1H, $J=1.7$ Hz), 11.37 (br s, 1H). Anal. ($C_{13}H_{11}Cl_2NO$) C, H, N.

6,8,9-Trichloro-1,2,3,4-tetrahydroacridine. A mixture of 6,8-Dichloro-1,2,3,4-tetrahydro-9-hydroxyacridine (1.00 g, 3.74 mmol) and $POCl_3$ (2.5 mL) was heated at 135 °C for 45 min. After cooling, the reaction mixture was poured into ice-water and neutralized with $NaHCO_3$. The suspension was extracted with Et_2O , and the organic layers, washed with brine, were dried and evaporated. The resulting residue, purified by crystallization from $EtOH$, afforded the title compound as a white solid. Yield 87%, mp 78-80 °C. 1H NMR ($CDCl_3$) δ 1.91-1.94 (m, 4H), 2.96 (m, 2H), 3.06 (m, 2H), 7.55 (d, 1H, $J=1.9$ Hz), 7.89 (d, 1H, $J=1.9$ Hz). Anal. ($C_{13}H_{10}Cl_3N$) C, H, N.

9-Azido-6,8-dichloro-1,2,3,4-tetrahydroacridine. To a solution of 6,8,9-trichloro-1,2,3,4-tetrahydroacridine (0.90 g, 3.16 mmol) in ethanol (10 mL), a solution of sodium azide (0.46 g, 7.16 mmol) in water (3 mL) was added; the reaction mixture was refluxed for 20 h, concentrated in vacuo, diluted with water and extracted with Et_2O . The combined organic extracts were dried and evaporated. The residue was purified by flash chromatography (hexane/ Et_2O , 8:2) to give the desired compound as a white powder. Yield 37%, mp 106-108 °C. 1H NMR ($CDCl_3$) δ 1.91-1.95 (m, 4H), 2.95-3.05 (m, 4H), 7.48 (d, 1H, $J=1.9$ Hz), 7.83 (d, 1H, $J=1.9$ Hz). Anal. ($C_{13}H_{10}Cl_2N_4$) C, H, N.

9-Amino-6,8-dichloro-1,2,3,4-tetrahydroacridine (2). To a suspension of $LiAlH_4$ (0.067g, 1.74 mmol) in 200 mL of dry Et_2O , a solution of 9-azido-6,8-dichloro-1,2,3,4-tetrahydroacridine (0.34 g, 1.16 mmol) in the same solvent was added at 0 °C with stirring. To reaction mixture was stirred under reflux for 2 h, and, after cooling to room temperature, Et_2O and crushed-ice were added. The mixture was extracted several times with Et_2O , and the organic extracts, dried and evaporated, afforded the desired product as a yellow powder, which was purified by flash chromatography ($CHCl_3$). Yield 55%, mp 182-184 °C. 1H NMR ($CDCl_3$) δ 1.77-1.80 (m, 4H), 2.44-2.48 (m, 2H), 2.75-2.97 (m, 2H), 6.57 (br s, 2H), 7.38 (d, 1H, $J=1.9$ Hz), 7.63 (d, 1H, $J=1.9$ Hz). Anal. ($C_{13}H_{12}Cl_2N_2$) C, H, N.

1,2,3,4-Tetrahydro-9-mercaptoacridine. A solution of KOH (3.00 g) in ethanol (20 mL) was bubbled with H₂S for 2 h; a solution of 9-chloro-1,2,3,4-tetrahydro-acridine (1.00 g) in ethanol was then added, and H₂S bubbled further for 5 h. The reaction mixture was poured into water and extracted with EtOAc. The combined organic layers, dried on Na₂SO₄, and concentrated in vacuo, afforded the desired compound as a yellow powder. Yield 96%, mp 246 °C. ¹H NMR (DMSO-d₆) δ 1.79-1.82 (m, 4H), 2.76-2.83 (m, 4H), 7.41-7.49 (m, 1H), 7.69-7.77 (m, 2H), 8.76 (d, 1H, *J*=8.2 Hz), 12.45 (br s, 1H). Anal. (C₁₃H₁₃NS) C, H, N.

Synthesis of compounds **3b** and **3c**

***N*-(6,8-Dichloro-1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine.** 1,2,3,4-tetrahydro-6,8,9-trichloroacridine (1.00 g, 3.490 mmol), heptane-1,7-diamine (1.36 g, 10.45 mmol) and 1-pentanol (5 mL) were heated under reflux (160 °C) for 10 h. After cooling to room temperature, the mixture was diluted with CH₂Cl₂ (50 mL) and then washed with 10% NaOH (1 x 50 mL) and water (2 x 40 mL). The organic layers were dried over Na₂SO₄, filtered, and concentrated in vacuo. The crude residue was purified by flash column chromatography (CHCl₃/CH₃OH/NH₃, 7:3:0.1) to afford the title compound as a colorless oil. Yield 63%. ¹H NMR (CDCl₃) δ 1.31-1.35 (m, 6H), 1.49-1.63 (m, 4H), 1.76-1.98 (m, 4H), 2.71 (t, 2H, *J*=6.1 Hz), 3.00 (t, 2H, *J*=6.4 Hz), 3.18 (t, 2H, *J*=6.9 Hz), 3.62 (t, 2H, *J*=6.5 Hz) 7.32 (d, 1H, *J*=1.9 Hz), 7.79 (d, 1H, *J*=1.9 Hz). Anal. C₂₀H₂₇Cl₂N₃.

***N,N*-Bis-(6,8-dichloro-1,2,3,4-tetrahydroacridin-9-yl)-1,7-diaminoheptane (**3b**).** *N*-(6,8-Dichloro-1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine (0.84 g, 2.21 mmol), 1,2,3,4-tetrahydro-6,8,9-trichloroacridine (1.26 g, 4.42 mmol) and 1-pentanol were reacted as above to afford **3b**. The residue, purified by flash column chromatography (CHCl₃/CH₃OH/NH₃, 9:1:0.01) and recrystallized from CH₂Cl₂/Et₂O, afforded **3b** as a yellow foam. Yield 60%, ¹H NMR (CDCl₃) δ 1.30 (m, 6H), 1.57-1.73 (m, 4H), 1.75-1.89 (m, 8H), 2.68 (t, 4H, *J*=6.1 Hz), 2.96 (t, 4H, *J*=6.3 Hz), 3.14 (t, 4H, *J*=6.8 Hz), 5.70 (br s, 2H), 7.29 (d, 2H, *J*=2.0 Hz), 7.76 (d, 2H, *J*=2.0 Hz). MS *m/z*: 631 (100), 595, 563, 361. Anal. C₃₃H₃₆Cl₄N₄: C, H, N.

***N*-(6,8-Dichloro-1,2,3,4-tetrahydroacridin-9-yl)-*N'*-(1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine (3c).** By reaction of *N*-(6',8'-Dichloro 1',2',3',4'-tetrahydroacridin-9'-yl)heptane-1,7-diamine (0.84g, 2.21 mmol), 1,2,3,4-tetrahydro-9-chloroacridine and 1-pentanol (5 mL), as described above, **3c** was obtained and purified by flash chromatography (CH₂Cl₂/CH₃OH/NH₃, 9:1:0.01), as yellow oil. Yield 63%. ¹H NMR (CDCl₃) δ 1.34-2.02 (m, 18H), 2.71-2.74 (m, 4H), 2.95-3.01 (m, 4H), 3.17 (t, 2H, *J*=6.8 Hz), 3.44 (t, 2H, *J*=6.7 Hz), 7.27-7.35 (m, 2H), 7.52 (t, 1H, *J*=7.1 Hz), 7.79 (d, 1H, *J*=1.8 Hz), 7.89 (t, 2H, *J*=8.5 Hz). MS *m/z*: 560, 524, 295, 281, 267, 225, 211, 198 (100). Anal. C₃₃H₃₈Cl₂N₄: C, H, N.

Synthesis of 3d, 3h, 3i.

7-Bromo *N*-(1,2,3,4-tetrahydroacridin-9-yl)heptan-1-amine. A solution of **1** (1.9 g, 9.6 mmol) in CH₃CN was added to powdered KOH (9.6 mmol) under argon. To the vigorously stirred mixture at room temperature, 1,7-dibromoheptane (9.6 mmol) was added. After stirring at room temperature for 12 h, the resulting mixture was poured into water and extracted with EtOAc (3x). The organic layer was dried over MgSO₄, filtered, concentrated in vacuo and purified by flash column chromatography (EtOAc/hexane/TEA, 6:4:2) to afford the title compound as a yellow oil. Yield 74%. ¹H NMR (CDCl₃) δ 1.21-1.41 (m, 6H), 1.62-1.80 (m, 2H), 1.83-1.92 (m, 6H), 2.70 (m, 2H), 3.05 (m, 2H), 3.34-3.51 (m, 4H), 3.9 (br s, 1H), 7.32 (t, 1H, *J*=7.6 Hz), 7.53 (t, 1H, *J*=7.0 Hz), 7.91 (t, 2H, *J*=7.6 Hz). MS *m/z*: 374, 295, 211, 197, 182 (100). Anal. C₂₀H₂₇BrN₂: C, H, N.

5-Bromo-*N*-(1,2,3,4-tetrahydroacridin-9-yl)pentan-1-amine (8). The title compound was prepared starting from **1** and 1,5-dibromopentane, using the procedure described above. Yield 73%. ¹H NMR (CDCl₃) δ 1.49-1.81 (m, 4H), 1.81-2.01 (m, 6H), 2.63-2.76 (m, 2H), 2.94-3.07 (m, 2H), 3.31-3.52 (m, 4H), 7.30-7.42 (m, 1H), 7.48-7.59 (m, 1H), 7.85-8.01 (m, 2H). Anal. C₁₈H₂₃BrN₂: C, H, N.

8-Bromo-*N*-(1,2,3,4-tetrahydroacridin-9-yl)octan-1-amine (9). The title compound was obtained starting from **1** and 1,8-dibromooctane, using the procedure described for **8**. Yield 67%. ¹H NMR (CDCl₃) δ 1.29-1.37 (m, 8H), 1.59-1.71 (m, 2H), 1.76-2.01 (m, 6H), 2.63-2.76 (m, 2H), 2.94-3.07

(m, 2H), 3.31-3.52 (m, 4H), 7.46-7.50 (m, 1H), 7.55-7.60 (m, 1H), 7.88-8.05 (m, 2H). MS m/z: 389, 309, 225, 211, 197, 183. Anal. C₂₁H₂₉BrN₂: C, H, N.

***N*-(1,2,3,4-tetrahydroacridin-9-yl)-7-[(1,2,3,4-tetrahydroacridin-9-yl)thio]heptan-1-amine**

(3d). A solution of 1,2,3,4-tetrahydro-9-mercaptoacridine (0.11 g, 0.53 mmol) in acetonitrile was added to powdered KOH (0.53 mmol) under argon. The mixture was vigorously stirred at room temperature and a solution of 7-Bromo-*N*-(1,2,3,4-tetrahydroacridin-9-yl)heptan-1-amine (0.20g, 0.53 mmol) in acetonitrile was added. The stirring was continued at room temperature for 4 h, and the reaction mixture was poured into water and extracted with EtOAc. The organic layers were dried and concentrated in vacuo; the residue, purified by flash column chromatography (EtOAc/hexane/TEA, 6:4:2), afforded **3d** as a clear yellow oil. Yield 84%. ¹H NMR (CDCl₃) δ 1.24-1.38 (m, 6H), 1.50-1.58 (m, 4H), 1.88-1.91 (m, 8H), 2.68 (m, 2H), 2.77 (t, 2H, J=6.9 Hz), 3.04 (m, 2H), 3.12-3.19 (m, 4H), 3.41 (t, 2H, J=7.0 Hz), 7.31 (t, 1H, J=7.2 Hz), 7.48-7.64 (m, 3H), 7.87-7.96 (m, 3H), 8.45 (d, 1H, J=8.8 Hz). MS m/z: 509, 327 (100), 312, 294, 281, 267, 237, 225, 211, 198, 183. Anal. C₃₃H₃₉N₃S: C, H, N.

***N*-(1,2,3,4-Tetrahydroacridin-9-yl)-8-[(1,2,3,4-tetrahydroacridin-9-yl)thio]octan-1-amine (3h).**

Following the procedure as above described for **3d**, **3h** was obtained as a yellow oil. Yield 62 %. ¹H NMR (CDCl₃) δ 1.25-1.32 (m, 8H), 1.47-1.64 (m, 4H), 1.90-1.92 (m, 8H), 2.69 (m, 2H), 2.78 (t, 2H, J=6.9 Hz), 2.81-3.23 (m, 6H), 3.43 (t, 2H, J=7.0 Hz), 7.32 (t, 1H, J=7.3 Hz), 7.49-7.57 (m, 3H), 7.90-7.95 (m, 3H), 8.46 (d, 1H, J=8.5 Hz). MS m/z: 523, 308 (100), 281, 267, 224, 214, 196, 183. Anal. C₃₄H₄₁N₃S: C, H, N.

***N*-(1,2,3,4-Tetrahydroacridin-9-yl)-5-[(1,2,3,4-tetrahydroacridin-9-yl)thio]pentan-1-amine**

(3i). The title compound was prepared following the procedure described for **3d**. After purification by flash column chromatography (EtOAc/hexane/TEA, 8:1:1), **3i** was obtained as a yellow oil. Yield 37%. ¹H NMR (CDCl₃) δ 1.49-1.62 (m, 6H), 1.88-1.98 (m, 8H), 2.62-2.78 (m, 4H), 3.12-3.27 (m, 6H), 3.38-3.44 (m, 2H), 7.21-7.35 (m, 1H), 7.41-7.69 (m, 3H), 7.81-8.01 (m, 3H), 8.48 (d, 1H, J=8.0 Hz). MS m/z: 481 (100), 284, 267, 225, 211, 198, 183. Anal. C₃₁H₃₅N₃S: C, H, N.

Synthesis of compounds 3e,f.

***N*-Methyl-*N*-[3-[(1,2,3,4-tetrahydroacridin-9-yl)amino]propyl]propane-1,3-diamine (6).**

Starting from 9-chloro-1,2,3,4-tetrahydroacridine and *N,N*-bis(3-aminopropyl)methylamine, using the procedure described for **3b**, and purification by flash chromatography (EtOAc/TEA, 7:3), yielded the title compound as a colorless oil. Yield: 35%. ¹H NMR (CDCl₃) δ 1.75-1.98 (m, 8H), 2.29 (s, 3H), 2.39-2.51 (m, 4H), 2.63-2.83 (m, 4H), 3.05-3.15 (m, 2H), 3.55-3.65 (m, 2H), 4.75 (br s, 2H), 5.08 (br s, 1H), 7.25-7.35 (m, 1H), 7.47-7.59 (m, 1H), 7.82-8.02 (m, 2H). Anal. C₂₀H₃₀N₄: C, H, N.

***N,N*-Bis[3-[(1,2,3,4-tetrahydroacridin-9-yl)amino]propyl]-*N*-methylamine (3e).** The title compound was prepared starting from 1,2,3,4-tetrahydro-9-chloroacridine and **6** as above described. After purification by flash chromatography (EtOAc/TEA, 8:2), **3e** was obtained as a colorless oil. Yield: 30%. ¹H NMR (CDCl₃) δ 1.75-1.98 (m, 12H), 2.30 (s, 3H), 2.51 (t, 4H, *J*=6.8 Hz), 2.60-2.71 (m, 4H), 2.99-3.10 (m, 4H), 3.55 (t, 4H, *J*=6.9 Hz), 7.18-7.29 (m, 2H), 7.45-7.55 (m, 2H), 7.85-7.99 (m, 4H). MS *m/z*: 508, 296 (100), 225, 212, 197. Anal. C₃₃H₄₁N₅: C, H, N.

***N*-[3-[(6,8-Dichloro-1,2,3,4-tetrahydroacridin-9-yl)amino]propyl]-*N*-[3-[(1,2,3,4-tetrahydroacridin-9-yl)amino]propyl]-*N*-methylamine (3f).** Similar to the procedure described for **3e**, the title compound was prepared starting from 6,8,9-trichloro-1,2,3,4-tetrahydroacridine and **6**. After purification by flash chromatography (EtOAc/TEA, 8:2), **3f** was obtained as a colorless oil. Yield: 20%. ¹H NMR (CDCl₃) δ 1.24-2.03 (m, 12H), 2.24 (s, 3H), 2.41-2.51 (m, 4H), 2.66-2.69 (m, 4H), 2.92-3.03 (m, 4H), 3.22-3.25 (m, 2H), 3.50-3.56 (m, 2H), 7.25-7.50 (m, 3H), 7.78-7.94 (m, 3H). MS *m/z*: 576 (100), 542, 296, 239, 225, 211, 199, 184, 168. Anal. C₃₃H₃₉Cl₂N₅: C, H, N.

Synthesis of 3g.

Bis[3-(*t*-butoxycarbonylamino)propyl]amine. Dry toluene (150 mL), *t*-butanol (40 mmol), KOH (1 mmol), and CDI (40 mmol) were added to a 250 mL round bottom flask with a dry N₂ inlet and magnetic stirrer and heated at 60 °C for 3 h. After addition of *N,N*-bis(3-aminopropyl)amine (20 mmol), the solution was stirred at 60°C for 3 h. The clear mixture was left to cool down,

concentrated in vacuo, dissolved in CH₂Cl₂, and washed three times with water. The obtained solution, dried on Na₂SO₄ and concentrated in vacuo, afforded the title compound as a colorless gum. Yield 96%. ¹H NMR (CDCl₃) δ 1.34 (s, 18H), 1.48-1.61 (m, 4H), 2.51-2.58 (m, 4H) 3.05-3.18 (m, 4H), 5.19 (br s, 2H). Anal. C₁₆H₃₃N₃O₄: C, H, N.

***N,N*-Bis[3-(*t*-butoxycarbonylamino)propyl]acetamide.** To a solution of the Bis[3-(*t*-butoxycarbonylamino)propyl]amine (300mg, 0.906 mmol) and TEA (1.087 mmol) in CH₂Cl₂ at 0°C, acetyl chloride (1.087 mmol) was added slowly. The mixture was stirred at room temperature for 2 h. Water was added, and the mixture was extracted with CH₂Cl₂. The organic layer, dried on Na₂SO₄, and evaporated, gave the desired product as a clear oil. Yield 95.6%. ¹H NMR (CDCl₃) δ 1.45 (s, 18H), 1.58-1.89 (m, 4H), 2.10 (s, 3H), 3.01-3.21 (m, 4H), 3.22-3.48 (m, 4H). Anal. C₁₈H₃₅N₃O₅: C, H, N.

***N,N*-Bis-[3-[(1,2,3,4-Tetrahydroacridin-9-yl)amino]propyl]acetamide (3g).** To a solution of the above described ester of carbamic acid (1.07 mmol) in CH₂Cl₂, trifluoroacetic acid (3 mL) was added and the mixture was stirred for 1 h at room temperature. The resulting solution was concentrated in vacuo, and the crude residue (0.97 mmol) was washed with ether. After that, triethylamine (0.97 mmol), 1,2,3,4-tetrahydro-9-chloroacridine (0.97 mmol) and pentanol (5 mL) were added, and the mixture heated to reflux (160 °C) for 6 h. Compound **7** was isolated, characterized and used in the next step. A solution of **7**, triethylamine (0.97 mmol), and 9-chloro-1,2,3,4-tetrahydroacridine (0.97 mmol) in pentanol (5 mL) was heated for 10 h with stirring. Following the same procedure described for **3e**, after purification by flash chromatography (EtOAc/CH₃OH/TEA, 1:1:1), **3g** was obtained as a yellow oil. Yield 30%. ¹H NMR (CDCl₃) δ 1.75-1.90 (m, 12H), 1.95 (s, 3H), 2.73 (m, 4H), 3.07 (m, 4H), 3.30-3.62 (m, 8H), 5.89 (br s, 2H), 7.33-7.37 (m, 2H), 7.49-7.53 (m, 2H), 7.89-7.97 (m, 4H). MS *m/z*: 535, 493, 352, 338, 225, 197, 182 (100). Anal. C₃₄H₄₁N₅O: C, H, N.

Molecular Modeling.

All the molecular modeling studies were performed on SGI Indigo II R10000 and SGI Octane 2XR10000 workstations.

Human AChE crystal structure and Human BuChE Homology model were downloaded from the PDB data bank (<http://www.rcsb.org/pdb/>; PDB IDs: 1B41, 1EVE, 1ACL, 1ACJ, 1E3Q, 1VOT, 1EA5, 2ACE, 2ACK, 1E66 and 1EHO). Hydrogens were added to all the PDB structures considering a pH value of 7. To introduce compounds **3e**, **3g** and **3h** in both hAChE (1B41) and BuChE (1EHO), their ligands (Fasciculin and (-) cocaine, respectively) were removed, using the unmerge command in the Biopolymer Module of Insight2000 (Accelrys, San Diego). An in depth analysis of the conserved water molecules in crystallized AChEs was performed (Insight2000 Homology module) to select those to include in subsequent calculations. On the basis of this inspection, all the water molecules present in 1B41 were maintained, with the exception of those, which would overlap the ligand, and one water molecule was added from 1ACJ (643H) active site. The same approach was used to add water molecules in the active site (as defined in the docking procedure) of 1EHO.

The newly designed compounds **3e**, **3g** and **3h** (considered in their protonated form) were built using the Insight 2000 Builder module. Since the subsequent docking procedure (Affinity module, Insight2000) requires reasonable initial structures, **3e**, **3g** and **3h** were subjected to a conformational search. Due to the high flexibility of the structures under study, their conformational space was sampled through 100 cycles of Simulated Annealing (Tripos force field, Sybyl software, Tripos, San Louis). A starting temperature of 1000 K was applied to surmount torsional barriers, the structure was held at that temperature for 1000 fs, then the temperature was reduced to 200 K by a decrement of 0.5K/fs. Resulting structures were subjected to an energy minimization protocol within the Insight2000 Discover module (CVFF force field¹, Steepest Descent algorithm; $\epsilon=80 \cdot r$) until the maximum RMS derivative was less than 0.001 kcal/Å, and subsequently ranked by their

conformational energy values. A visual inspection of the low energy resulting conformers, placed in the 1B41 and 1EHO active sites, led to the selection of each starting ligand-protein complex. Ligands were oriented in the active sites by placing the/a tetrahydroaminoacridine moiety in the position corresponding to that occupied by tacrine in *Torpedo Californica* AChE catalytic site (PDB ID: 1ACJ). Obtained complexes were subjected to preliminary energy minimization (CVFF force field, Steepest Descent algorithm; $\epsilon=80 \cdot r$) until the maximum RMS derivative was less than 0.5 kcal/Å, to generate roughly docked starting structures, as required by Affinity docking procedure. Successively, flexible docking was achieved through the Affinity module in the Insight2000 suite, using the SA_Docking procedure² and the Cell_Multipole³ method for nonbond interactions. A binding domain area was defined as a subset including all the residues having at least one atom within 5 Å radius from any given ligand atom. All the atoms included in the binding domain area were left free to move during the entire docking calculations. A Monte Carlo/minimization approach for random generation of a maximum of twenty structures was used, with an energy tolerance of 10^6 Kcal/Mol to ensure a wide variance of the input structures to be minimized (2500 iterations; CVFF force field, $\epsilon=80 \cdot r$). During this step the ligand is moved by a random combination of translation, rotation, and torsional changes, to sample both the conformational space of the ligand and its orientation with respect to the receptor. van der Waals (vdW) and Coulombic terms were scaled to a factor of 0.1 to avoid very severe divergences in the Coulombic and vdW energies. The Metropolis test, at a temperature of 310 K, and a structure similarity check (RMS tolerance = 0.3 kcal/Å), were applied to select acceptable structures. Fifty stages of Simulated Annealing (100 fs each) were applied on the resulting complexes. Over the course of the Simulated Annealing, system temperature was linearly decreased from 500 K to 300 K, concurrently the Van Der Waals and Coulombic scale factors were similarly decreased from their initial values (defined above as 0.1) to their final value (1.0). A final round of 10^6 minimization steps was applied at the end of the molecular dynamics.

After the docking procedure, the resulting complexes needed to be further minimized (CVFF forcefield, $\epsilon=80 \times r$) by a combination of Steepest Descent (maximum RMS derivative less than 0.1 Kcal/Å) and Conjugate Gradients algorithms (maximum RMS derivative less than 0.01 Kcal/Å).

Measurement of AChE/BuChE Activity

The biological activity of the new homo- and heterobivalent analogues of THA (THA-An) were evaluated using purified FBS AChE and Eq BuChE. AChE and BuChE activities were measured in 50 mM sodium phosphate, pH 8.0, at 25°C as described using acetylthiocholine (ATC) and butyrylthiocholine (BTC) as substrates, respectively.⁴ Inhibition of enzyme activity was measured in 50 mM sodium phosphate, pH 8.0, over a substrate concentration range of 0.01 - 30 mM and at least six inhibitor concentrations to determine the components of competitive and non-competitive inhibition. Plots of initial velocities versus substrate concentrations at a series of inhibitor concentrations were analyzed by non-linear least squares methods to determine the values of K_m (Michaelis-Menten constant) and V_{max} (maximal velocity). Non-linear regression analysis of the plots of V_{max} / K_m values versus [THA-An] were used for the determination of K_i values. The K_i values for the inhibition of FBS AChE and Eq BuChE by the various inhibitors are reported in Table 1.

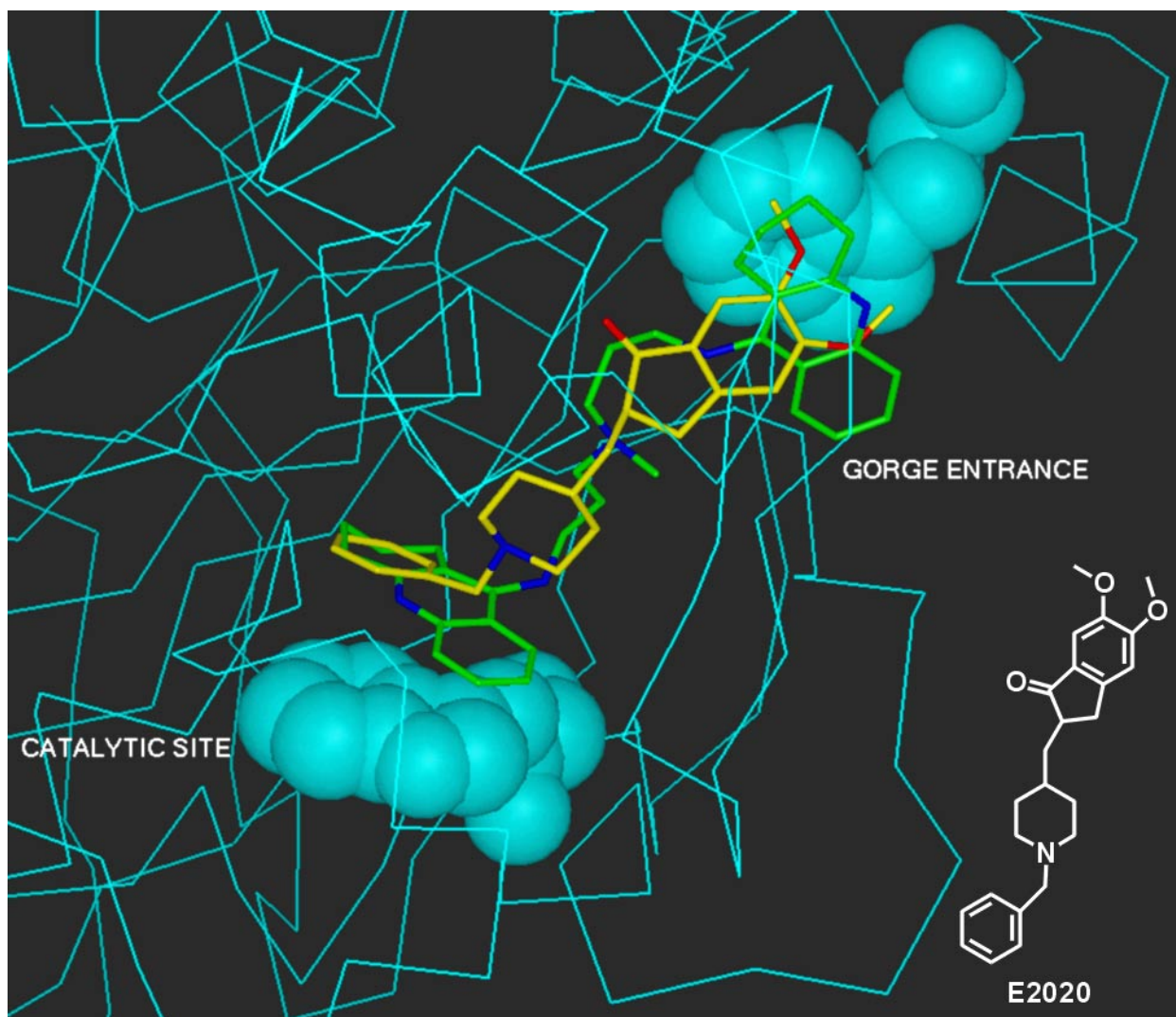


Figure 4. Comparison of the binding mode of E2020 (yellow) in *Torpedo californica* AChE (PDB ID 1EVE)⁵ and that of **3e** (coloured by atom type) docked into hAChE, obtained by the superimposition of the backbone atoms of the Trp residues in the PAS and catalytic site of the two AChEs. E2020 heteroatoms are highlighted in blue (nitrogen) and red (oxygen). hAChE α -carbons and the van der Waals volume of W86 and W286 are displayed in cyan. Hydrogens are omitted for clarity.

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Table. Elemental analysis values of the new compounds.

Compound	Formula	C%		H%		N%	
		calc.	found	calc.	found	calc.	found
2	C ₁₃ H ₁₂ Cl ₂ N ₂	58.45	58.43	4.53	4.59	10.49	10.41
3a	C ₃₂ H ₃₈ N ₄	80.29	80.33	8.00	7.95	11.70	11.76
3b	C ₃₃ H ₃₆ Cl ₄ N ₄	62.87	62.84	5.76	5.79	8.89	8.85
3c	C ₃₃ H ₃₈ Cl ₂ N ₄	70.58	70.61	6.82	6.86	9.98	9.93
3d	C ₃₃ H ₃₉ N ₃ S	77.75	77.70	7.71	7.75	8.24	8.21
3e	C ₃₃ H ₄₁ N ₅	78.07	78.11	8.14	8.10	13.79	13.83
3f	C ₃₃ H ₃₉ Cl ₂ N ₅	68.74	68.72	6.82	6.89	12.15	12.09
3g	C ₃₄ H ₄₁ N ₅ O	76.23	76.20	7.71	7.76	13.07	13.11
3h	C ₃₄ H ₄₁ N ₃ S	77.97	78.01	7.89	7.83	8.02	8.09
3i	C ₃₁ H ₃₅ N ₃ S	77.30	77.35	7.32	7.27	8.72	8.68
6	C ₂₀ H ₃₀ N ₄	73.58	73.56	9.26	9.32	17.16	17.20
8	C ₁₈ H ₂₃ BrN ₂	62.25	62.19	6.68	6.63	8.07	8.04
9	C ₂₁ H ₂₉ BrN ₂	64.78	64.81	7.51	7.48	7.19	7.13