# Supporting information

## Synthesis of monomeric derivatives to probe memoquin's bivalent interactions

*Maria Laura Bolognesi,* GianPaolo Chiriano, Manuela Bartolini, Francesca Mancini, Giovanni Bottegoni, Valentina Maestri, Stefan Czvitkovich, Manfred Windisch, Andrea Cavalli, Anna Minarini, Michela Rosini, Vincenzo Tumiatti, Vincenza Andrisano and Carlo Melchiorre*

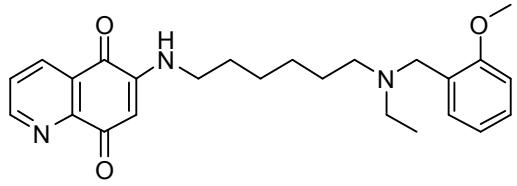
**Contents**

## Chemistry

### *General chemical methods*

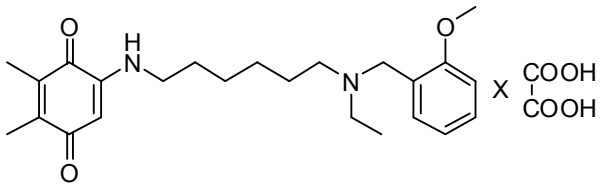
Melting points were taken in glass capillary tubes on Buchi SMP-20 apparatus and are uncorrected. Direct infusion ESI-MS spectra were recorded on Perkin-Elmer 297 and Waters ZQ 4000 apparatus, respectively. NMR spectra were recorded on Varian VXR 200, 300 and MR 400 instruments. Chemical shifts are reported in parts per millions (ppm) relative to tetramethylsilane (TMS). 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) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F254), then visualized in an iodine chamber or with an UV lamp. The term "dried" refers to the use of anhydrous sodium sulphate. Compounds were named following IUPAC rules as applied by Beilstein-Institute AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry.

**6-(6-(ethyl(2-methoxybenzyl)amino)hexylamino)quinoline-5,8-dione (3).**



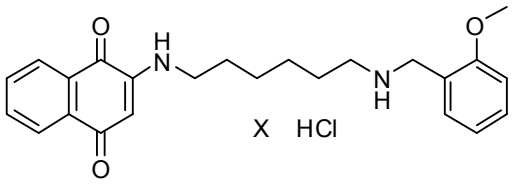
A suspension of **13**[1] (0.20 g, 1.06 mmol) in EtOH (6 mL) was added dropwise to a solution of **10**[2, 3] (0.15 g, 0.57 mmol) in EtOH (6 mL). The resulting reaction mixture was stirred at reflux for 4 h and at room temperature over the night. Then, the solvent was evaporated and the obtained violet crude product was purified by flash chromatography ($CH_2Cl_2$/MeOH/aqueous 33% ammonia = 9/1/0.05). Pure **3** (0.22 g) was obtained as a violet oil. Yield 92%; ESI-MS (m/z): 422 ($M+H^+$); $^1$H-NMR ($CDCl_3$, 300 MHz): $\delta$ 9.05 (m, 1H), 8.40 (d, $J$ = 7.5, 1H), 7.63-7.59 (m, 2H), 7.41-7.33 (m, 1H), 7.04 (t, $J$ = 7.2, 1H), 6.93 (d, $J$ = 8.4, 1H), 6.00 (br s exch, 1H), 5.95 (s, 1H), 4.01 (s, 2H), 3.89 (s, 3H), 3.23 (q, $J$ = 6.6, 2H), 2.97-2.65 (m complex, 4H), 1.82-1.70 (m, 4H), 1.45-1.22 (m, 7H); $^{13}$C-NMR ($CDCl_3$, 100 MHz): $\delta$ 181.5, 181.1, 157.7, 155.0, 149.2, 147.5, 134.1, 130.8, 128.5, 127.2, 126.1, 120.5, 110.3, 101.7, 55.4, 52.8, 50.9, 47.5, 42.5, 28.0, 26.9, 26.7, 26.0, 11.0. Anal. calcd. for $C_{25}H_{31}N_3O_3$ (421.53): C, 71.23; H, 7.41; N, 9.97. Found: C, 71.03; H, 7.57; N, 9.79.

**5-(6-(ethyl(2-methoxybenzyl)amino)hexylamino)-2,3-dimethylcyclohexa-2,5-diene-1,4-dione oxalate (4).**



Quinone **14** (0.20 g, 1.47 mmol) was added to a solution of **10**[2, 3] (0.39 g, 1.47 mmol) in $CH_2Cl_2$ (5 mL). The reaction mixture was stirred for 3 h at room temperature in an open flask. In the reaction mixture, a change of color from yellow to red was observed. The solvent was evaporated under vacuum, and the crude red oil obtained was purified by flash cromatography (EtOAc/petroleum ether/MeOH/aqueous 33% ammonia = 8/2/0.1/0.01). **4** (0.50 g; 1.25 mmol) was then dissolved in EtOH (7 mL) and to this solution a solution of oxalic acid (0.11 g, 1.26 mmol) in $Et_2O$ (5 mL) was added. The formed solid was collected by filtration, washed by $Et_2O$ and petroleum ether, and dried to afford 0.50 g of **4** as oxalate salt. Yield 70%; mp = 106 °C; ESI-MS (m/z): 399 ($M+H^+$); $^1$H-NMR ($CDCl_3$, free base, 300 MHz): $\delta$ 7.44 (d, $J$ = 7.6, 1H), 7.24 (t, $J$ = 8.0, 1H), 6.97 (t, $J$ = 7.2, 1H), 6.89 (d, $J$ = 8.0, 1H), 5.62 (br s exch, 1H), 5.36 (s, 1H), 3.87 (s, 3H), 3.64 (s, 2H), 3.07 (q, $J$ = 6.6, 2H), 2.62-2.49 (m, 4H), 2.08 (s, 3H), 2.01 (s, 3H), 1.87-1.58 (m complex, 8H), 1.10 (t, $J$ = 6.6, 3H); $^{13}$C-NMR ($CD_3OD$, 100 MHz): $\delta$ 187.2, 184.6, 166.6, 159.6, 148.9, 145.1, 137.8, 133.6, 133.3, 122.2, 119.2, 112.4, 97.0, 56.2, 53.7, 53.1, 49.5, 43.0, 28.7, 27.5, 27.3, 24.5, 12.8, 11.8, 8.9. Anal. calcd. for $C_{26}H_{36}N_2O_7$ (488.57): C, 63.92; H, 7.43; N, 5.73. Found: C, 63.73; H, 7.56; N, 5.67.

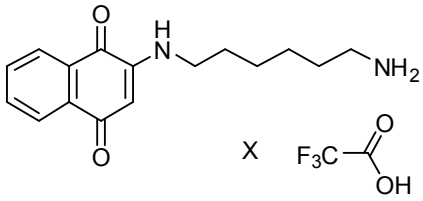
**2-(6-(2-Methoxybenzylamino)hexylamino)naphthalene-1,4-dione hydrochloride (6).**



The naphthalene-1,4-dione (0.35 g, 2.20 mmol) was suspended in MeOH (6 mL). To this suspension, a solution of **11**[3] (0.52 g, 2.20 mmol) in MeOH (6 mL) was added dropwise. The resulting reaction mixture was stirred at room temperature for 4 h in an open flask. The solvent was evaporated under vacuum, and the final crude product was purified by gravity chromatography ($CH_2Cl_2$/EtOH/aqueous 33% ammonia = 9/1/0.05). 0.14 g of free base were obtained as a reddish solid, which was transformed in the corresponding hydrochloride salt (**6**). Yield 16%; mp = 160-164 °C; ESI-MS (m/z): 393 ($M+H^+$); $^1$H-NMR ($CDCl_3$, 400 MHz, free base): $\delta$ 8.11 (d, $J$ = 7.6, 1H), 8.04 (d, $J$ = 7.6, 1H), 7.71 (t, $J$ = 8.0, 1H), 7.62 (t, $J$ = 7.2, 1H), 7.26-7.22 (m, 2H), 6.93-6.86 (m, 2H), 5.88 (br s exch, 1H), 5.72 (s, 1H), 3.84 (s, 3H), 3.81 (s, 2H), 3.16 (q, $J$ = 6.8, 2H), 2.61 (t, $J$ = 6.8, 2H), 2.28 (br s exch, 1H), 1.70-1.67 (m, 2H), 1.57-1.53 (m, 2H), 1.40-1.38 (m, 4H); $^{13}$C-NMR ($CDCl_3$, 100 MHz, free base): $\delta$ 192.8, 191.9, 157.6, 147.9, 134.7, 133.7, 131.9, 130.5, 130.0, 128.4, 127.6, 126.2,

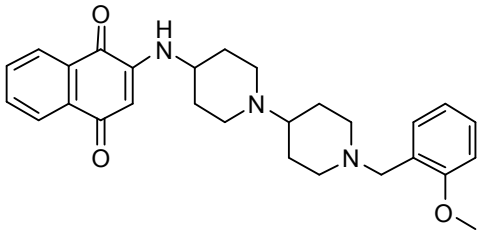
126.1, 120.4, 110.2, 100.7, 55.2, 49.0, 48.7, 42.5, 29.6, 28.1, 26.9, 26.8. Anal. calcd. for $C_{24}H_{29}ClN_2O_3$ (428.95): C, 67.20; H, 6.81; N, 6.53. Found: C, 66.98; H, 6.73; N, 6.40.

**2-(6-Aminohexylamino)naphthalene-1,4-dione trifluoroacetate (7).**



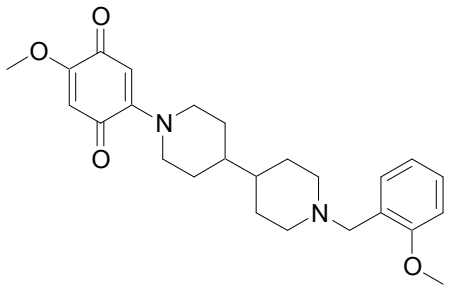
To a solution cooled in an ice bath of **15** (0.038g, 0.102 mmol) in $CHCl_3$ (3 mL) TFA (0.35 g, 3.06 mmol) was added dropwise. The mixture was stirred for 5 h at room temperature, and then was washed with $Et_2O$ (3 x 5 mL). The precipitate was filtered and the pure compound **7** was obtained as orange solid (0.025 g). Yield 63%; mp = 156 °C; ESI-MS (m/z): 273 ($M+H^+$); $^1$H-NMR (DMSO, 400 MHz) $\delta$ 7.97 (d, $J$ = 7.2, 1H), 7.93 (d, $J$ = 7.6, 1H), 7.82 (t, $J$ = 7.2, 1H), 7.72 (t, $J$ = 7.2, 1H), 7.68 (br s exch, 3H), 7.58 (t, $J$ = 7.2, 1H), 5.67 (s, 1H), 3.17 (q, $J$ = 7.2, 2H), 2.78-2.76 (m, 2H), 1.59-1.54 (m, 4H), 1.38-1.36 (m, 4H); $^{13}$C-NMR (DMSO, 100 MHz) $\delta$ 182.0, 181.6, 158.4, 158.1, 149.0, 135.3, 133.6, 132.6, 130.8, 126.3, 125.7, 99.6, 42.2, 27.5, 27.3, 26.4, 25.9. Anal. calcd. for $C_{18}H_{21}F_3N_2O_4$ (386.37): C, 55.96; H, 5.48; N, 7.25. Found: C, 55.82; H, 5.57; N, 7.02.

**2-(1'-(2-Methoxybenzyl)-1,4'-bipiperidin-4-ylamino)naphthalene-1,4-dione (8).**



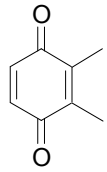
The naphthalene-1,4-dione (0.06 g, 0.39 mmol) was suspended in MeOH (2.5 mL). To this suspension, a solution of **18** (0.12 g, 0.39 mmol) in MeOH (2.5 mL) was added dropwise. The resulting reaction mixture was stirred at room temperature for 5 h in an open flask. The precipitate obtained was filtered and dried under vacuum. Compound **8** (0.075 g) was obtained as a brown solid. Yield 42%; mp = 102-105 °C; ESI-MS (m/z): 460 ($M+H^+$); $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 8.08 (d, $J$ = 7.6, 1H), 8.03 (d, $J$ = 7.6, 1H), 7.71 (t, $J$ = 7.2, 1H), 7.60 (t, $J$ = 7.2, 1H), 7.37-7.35 (m, 1H), 7.23 (t, $J$ = 7.6, 1H), 6.93 (t, $J$ = 7.6, 1H), 6.86 (d, $J$ = 8.4, 1H), 5.82-5.80 (m, 1H), 5.74 (s, 1H), 3.81 (s, 3H), 3.60 (br s, 2H), 3.30-3.26 (m, 1H), 3.04-3.02 (m, 2H), 2.92-2.89 (m, 2H), 2.37-2.32 (m, 3H), 2.08-2.04 (m, 4H), 1.77-1.57 (m, 6H); $^{13}$C-NMR ($CDCl_3$, 100 MHz): $\delta$ 183.2, 183.1, 158.0, 146.9, 135.0, 133.5, 132.2, 130.8, 128.3, 126.5, 126.4, 120.6, 110.7, 101.3, 94.7, 55.9, 55.7, 53.3, 49.9, 47.9, 46.1, 31.7, 28.1. Anal. calcd. for $C_{28}H_{33}N_3O_3$ (459.58): C, 73.18; H, 7.24; N, 9.14. Found: C, 73.04; H, 7.36; N, 8.97.

**2-methoxy-5-(1'-(2-methoxybenzyl)-4,4'-bipiperidin-1-yl)cyclohexa-2,5-diene-1,4-dione (9).**



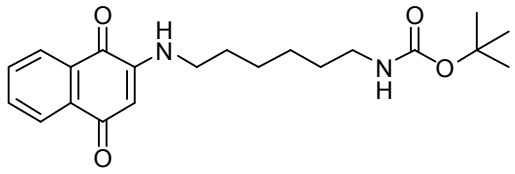
A solution of diamine **19** (1.20 g, 4.16 mmol) in $CHCl_3$ was added dropwise to a suspension of 2,5-dimethoxycyclohexa-2,5-diene-1,4-dione (0.10 g, 0.59 mmol) in $CHCl_3$ (50 mL). The resulting reaction mixture, while stirred at reflux for 72 h became progressively red. After cooling, the solvent was evaporated under vacuum. The crude mixture was purified by flash cromatography ($CH_2Cl_2$/MeOH/aqueous 33% ammonia = 9.5/0.5/0.05) and compound **9** (0.06 g) was obtained as a red solid. Yield 24%; m.p. = 182 °C; ESI-MS (m/z): 425 ($M+H^+$); $^1$H-NMR ($CD_3OD$, 400 MHz): $\delta$ 7.21-7.17 (m, 2H), 6.88 (d, $J$ = 8.8, 1H), 6.82 (t, $J$ = 7.2, 1H), 5.70 (s, 1H), 5.56 (s, 1H), 4.08 (d, $J$ = 12.8, 2H), 3.73 (s, 3H), 3.69 (s, 3H), 3.57 (s, 2H), 2.95 (d, $J$ = 11.6, 2H), 2.87 (t, $J$ = 12.4, 2H), 2.11-1.99 (m, 2H), 1.72 (d, $J$ = 12.4, 2H), 1.64 (d, $J$ = 12.8, 2H), 1-35-1.19 (m complex, 6H); $^{13}$C-NMR ($CD_3OD$, 100 MHz): $\delta$ 184.3, 179.9, 160.1, 158.2, 153.1, 131.5, 129.0, 119.8, 110.3, 105.3, 102.6, 55.7, 55.5, 54.3, 53.2, 49.5, 40.3, 39.9, 29.2, 28.05. Anal. calcd. for $C_{25}H_{32}N_2O_4$ (424.53): C, 70.73; H, 7.60; N, 6.60. Found: C, 70.60; H, 7.71; N, 6.46.

**2,3-dimethylcyclohexa-2,5-diene-1,4-dione (14).**



The $MnO_2$ (2.51 g, 28.87 mmol) was carefully added to a solution of the 2,3-dimethylbenzene-1,4-diol (0.50 g, 3.62 mmol) in dry $Et_2O$ (50 mL). The resulting reaction mixture was stirred at room temperature for 4 h and then filtered through celite. The solvent was evaporated under vacuum affording compound **14** (0.39 g) as a yellow solid. Yield 79%. m.p. = 53-54 °C (lit.[4] 54-55 °C); $^1$H-NMR ($CDCl_3$, 300 MHz): $\delta$ 2.07 (s, 6H), 6.75 (s, 2H).

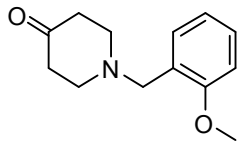
**Tert-butyl 6-(1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)hexylcarbamate (15).**



To a solution of naphthalene-1,4-dione (0.05 g, 0.32 mmol) in MeOH (1 mL) a solution of *tert*-butyl 6-aminohexylcarbamate, **12**, (0.136 g, 0.63 mmol) in MeOH (2 mL) was added. The reaction mixture was stirred at room temperature over the night in an open flask and, then, cooled in an ice bath. After adding water and ice to the reaction mixture, a red precipitate was obtained. The mixture was stirred for 1 h at room temperature and, then, filtered. Subsequently, the filtered solid was crystallized in EtOH (2 mL), obtaining the pure compound **15** as an orange solid (0.038 g). Yield 32%; $^1$H-NMR ($CDCl_3$, 400 MHz) $\delta$ 8.09-8.05 (m, 2H), 7.73-

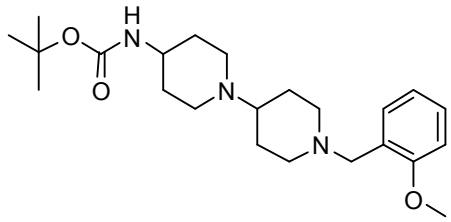
7.69 (m, 1H), 7.65-7.60 (m, 1H), 5.91 (br s exch, 1H), 5.72 (s, 1H), 4.55 (br s exch, 1H), 3.17-3.12 (m, 4H), 1.73-1.67 (m, 4H), 1.60-1.44 (m, 13H)

**1-(2-Methoxybenzyl)piperidin-4-one (16).**



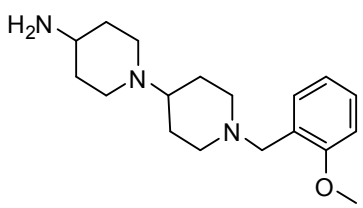
This compound was synthesized by a procedure different from that reported before.[5] 1-(Chloromethyl)-2-methoxybenzene (2.00 g, 12.82 mmol), piperidin-4-one monohydrate hydrochloride (2.20 g, 12.82 mmol), $K_2CO_3$ (3.54 g, 25.60 mmol) and KI (0.21 g, 1.28 mmol) were suspended in dry DMF (20 mL). The resulting reaction mixture was stirred at reflux temperature for 4 h. The solvent was evaporated under vacuum, and the final crude product was purified by flash chromatography ($CH_2Cl_2$/MeOH = 9.5/0.5). Pure **16** (2.31 g) was obtained as a foam solid. Yield 82%; $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 7.39 (d, $J$ = 7.6, 1H), 7.24 (t, $J$ = 8.0, 1H), 6.95 (t, $J$ = 7.2, 1H), 6.87 (d, $J$ = 8.0, 1H), 3.82 (s, 3H), 3.68 (s, 2H), 2.80 (t, $J$ = 6.4, 4H), 2.45 (t, $J$ = 6.4, 4H).

**Tert-butyl 1'-(2-methoxybenzyl)-1,4'-bipiperidin-4-ylcarbamate (17).**



Compound **16** (0.40 g, 1.82 mmol) was dissolved in dry THF (4 mL). To this solution, *tert*-butyl piperidin-4-ylcarbamate (0.36 g, 1.82 mmol), $(CH_3COO)_3BHNa$ (0.46 g, 2.18 mmol) and then $CH_3COOH$ (0.15 g, 2.50 mmol) were added. The resulting reaction mixture was stirred at room temperature under the $N_2$ flux over the night. The solvent was evaporated under vacuum, and the residue was washed with a saturated solution of $Na_2CO_3$, extracted with $CH_2Cl_2$ (3 x 10mL). The combined organic layers were dried and concentrated under vacuum. The final crude product was purified by gravity chromatography ($CH_2Cl_2$/MeOH = 9/1). Compound **17** (0.46 g) was obtained as a foam solid. Yield 63%; $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 7.35 (d, $J$ = 7.6, 1H), 7.21 (t, $J$ = 7.6, 1H), 6.92 (t, $J$ = 7.2, 1H), 6.85 (d, $J$ = 7.6, 1H), 4.43 (br s exch, 1H), 3.81 (s, 3H), 3.54 (s, 2H), 3.42 (br s, 1H), 2.98-2.97 (m, 2H), 2.86-2.83 (m, 2H), 2.30-2.24 (m, 3H), 2.05-1.98 (m, 2H), 1.94-1.91 (m, 2H), 1.75-1.72 (m, 2H), 1.65-1.58 (m, 2H), 1.44 (s, 9H), 1.41-1.38 (m, 2H).

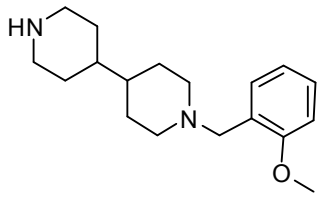
**1'-(2-Methoxybenzyl)-1,4'-bipiperidin-4-amine (18).**



Compound **17** (1.80 g, 4.46 mmol) was dissolved in dry $CH_2Cl_2$ (8 mL). To this solution cooled in an ice bath, TFA (10.17 g, 89.20 mmol) was added dropwise. The resulting reaction mixture was stirred at room temperature for 5 h. The solvent was evaporated

under vacuum. The obtained yellow oil was dissolved in $H_2O$ (5 mL). The resulting aqueous layer was washed with $Et_2O$, basified with a 2N solution of NaOH, and then extracted with $CH_2Cl_2$ (3 x 10 mL). The combined organic layers were dried and concentrated under vacuum. Pure **18** (0.94 g) was obtained as a white foam. Yield 69%; $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 7.34 (d, $J$ = 7.6, 1H), 7.21 (t, $J$ = 7.6, 1H), 7.01 (t, $J$ = 7.2, 1H), 6.85 (d, $J$ = 7.6, 1H), 3.80 (s, 3H), 3.54 (s, 2H), 2.99-2.96 (m, 2H), 2.88-2.85 (m, 2H), 2.63-2.59 (m, 1H), 2.32-2.18 (m, 3H), 2.04-1.99 (m, 2H), 1.91 (br s exch, 2H), 1.82-1.80 (m, 2H), 1.74-1.72 (m, 2H), 1.62-1.46 (m, 2H), 1.38-1.34 (m. 2H).

**1-(2-methoxybenzyl)-4,4'-bipiperidine (19).**




This compound was synthesized by a procedure different from that reported before.[6] To a solution of 4,4'-bipiperidine dihydrochloride (2.41 g, 10.0 mmol) in MeOH (50 mL) KOH (0.15 g, 2.72 mmol) and, subsequently, 2-methoxybenzaldehyde (1.05 g, 7.69 mmol) were added. The resulting reaction mixture was stirred at reflux for 15 min and after cooling in an ice bath, $NaBH_3CN$ (0.18 g, 2.90 mmol) was added. The resulting mixture was stirred at room temperature for 3 h. The solvent was evaporated under vacuum. The obtained residue was dissolved in $H_2O$ (40 mL). The resulting solution was acidified with HCl 6N and extracted with $Et_2O$ (3 x 20 mL). The aqueous phase, after basification with NaOH 40%, was extracted with $CHCl_3$ (3 x 40 mL). The combined organic layers were dried and concentrated under vacuum. The final crude product was purified by flash chromatography ($CH_2Cl_2$/MeOH/aqueous 33% ammonia = 6.5/3.5/0.35). Pure **19** (1.20 g) was obtained as a white solid. Yield 54%; mp = 120 °C; $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 7.66 (d, $J$ = 7.2, 1H), 7.40 (t, $J$ = 8.0, 1H), 7.02 (t, $J$ = 7.2, 1H), 6.93 (d, $J$ = 8.0, 1H), 4.23 (s, 2H), 3.85 (s, 3H), 3.51-3.43 (m, 4H), 2.83 (t, $J$ = 11.6, 2H), 2.68 (t, $J$ = 12.0, 2H), 2.25 (br s, 1H), 2.06-1.97 (m, 2H), 1.87-1.84 (m, 4H), 1.77-1.71 (m, 2H), 1.48-1.46 (m, 1H), 1.38-1.36 (m, 1H); $^{13}$C-NMR ($CDCl_3$, 100 MHz): $\delta$ 157.6, 130.5, 127.8, 126.4, 120.2, 110.3, 56.3, 55.4, 55.3, 46.4, 41.0, 40.8, 29.6, 29.3.

**Biological Methods**

***Enzymatic assays procedures***

**Inhibition of BACE-1 activity.** Purified Baculovirus-expressed BACE-1 (β-secretase) in 50 mM Tris (pH = 7.5), 10% glycerol and rhodamine-derivative substrate (Panvera peptide) were purchased from Invitrogen (Milan, Italy). Sodium acetate and DMSO were from Sigma Aldrich (Milan, Italy). Purified water from Milli-RX system (Millipore, Milford, MA, USA) was used to prepare buffers

and standard solutions. Spectrofluorometric analyses were carried out on a Fluoroskan Ascent multiwell spectrofluorometer (excitation: 544 nm; emission: 590 nm) by using black microwell (96 wells) Corning plates (Sigma Aldrich, Milan, Italy).

Stock solutions of the tested compounds were prepared in DMSO and diluted with 50 mM sodium acetate buffer pH = 4.5.

Specifically, 20 μL of BACE-1 enzyme (11.7 nM, final concentration) were incubated with 20 μL of test compound for 60 min. To start the reaction, 20 μL of Panvera peptide (0.25 μM, final concentration) were added to each well. The mixture was incubated at 37 °C for 60 min. To stop the reaction, 20 μL of BACE-1 stop solution (sodium acetate 2.5 M) were added to each well. Then the spectrofluorometric assay was performed by reading the fluorescence signal at 590 nm.

The DMSO concentration in the final mixture maintained below 5% (v/v) guaranteed no significant loss of enzyme activity.The fluorescence intensities with and without inhibitor were compared and the percent inhibition due to the presence of test compounds was calculated. The background signal was measured in control wells containing all the reagents, except BACE-1 and subtracted. The % inhibition due to the presence of increasing test compound concentration 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 BACE-1 in the presence and in the absence of inhibitor, respectively. Inhibition curves were obtained by plotting the % inhibition versus the logarithm of inhibitor concentration in the assay sample, when possible. The linear regression parameters were determined and the $IC_{50}$ extrapolated (GraphPad Prism 4.0, GraphPad Software Inc.). For **2**, inhibition curve was obtained by plotting the % inhibition versus the logarithm of inhibitor concentration, (range 0.5-6 μM) in the assay sample and the IC50 value extrapolated (n=3)".

To demonstrate inhibition of BACE-1 activity a peptido-mimetic inhibitor (β-secretase inhibitor IV, Calbiochem) was serially diluted into the reactions' wells ($IC_{50}$ = 0.013 μM).

For **1** and **2**, additional experiments were performed by using the following procedure:[7] 5 μL of test compounds (or DMSO, if preparing a control well) were pre-incubated with 175 μL of enzyme (BACE-1, Sigma in 20 mM sodium acetate containing CHAPS 0.1% w/v)[8,9] for 1 hour at room temperature. The substrate (methoxycoumarin-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys-dinitrophenyl, M-2420, Bachem, Germany) was then added (3 μM, final concentration) and left to react for 15 min. The fluorescence signal was read at $\lambda_{em}$ = 405 nm ($\lambda_{exc}$ = 320 nm).

**Inhibition of AChE and BuChE activities.** The method of Ellman et al. was followed.[10] Five different concentrations of each compound were selected in order to obtain inhibition of AChE or BuChE activities comprised between 20 and 80%. The assay solution consisted of a 0.1 M

potassium 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 BuChE from human serum (Sigma Chemical), and 550 μM of substrate (acetylthiocholine iodide or butyrylthiocholine iodide, respectively). Test compounds were added to the assay solution and preincubated at 37 °C with the enzyme for 20 min before the addition of substrate. Enzyme reaction was followed at 412 nm for five min by a double beam spectrophotometer (Jasco V-530). Assays were carried out with a blank containing all components except AChE or BuChE 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 inhibition curves (percent inhibition *vs* log inhibitor concentration).

**Inhibition of AChE-induced Aβ-amyloid aggregation.** Aliquots of 2 μL $A\beta_{40}$ peptide (Bachem AG, Germany), lyophilized from a 2 mg $mL^{-1}$ HFIP (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 human recombinant AChE (final concentration 2.30 μM, Aβ/AChE molar ratio 100:1) and AChE in the presence of 2 μL of tested inhibitor (final concentration = 100 μM) were added. Blanks containing Aβ, AChE, and Aβ plus inhibitor, 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 fluorescence method was then applied.[11-13]
Analyses were performed with a Jasco Spectrofluorometer FP-6200 using a 3 ml quartz cell. After incubation, the samples containing Aβ, or Aβ plus AChE, or Aβ plus AChE in the presence of inhibitors were diluted with 50 mM glycine-NaOH buffer (pH 8.5) containing 1.5 μM thioflavin T to a final volume of 2.0 mL. A 300s-time scan of the emitted fluorescence ($\lambda_{exc}$=446 nm, $\lambda_{em}$=490 nm) was performed and the intensity values at the plateau were averaged after subtracting the background fluorescence of 1.5 μM thioflavin T and AChE.
The fluorescence intensities in the presence and in the absence of inhibitor were compared and the percentage of inhibition 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.[11]

**Inhibition of $A\beta_{42}$ self-aggregation**. HFIP pretreated $A\beta_{42}$ samples (Bachem AG, Switzerland) were resolubilized with a $CH_3CN$/0.3 mM $Na_2CO_3$/250 mM NaOH (48.4/48.4/3.2) mixture in order to have a stable stock solution ([Aβ] = 500 μM). Experiments were performed by diluting the

peptide stock solution in 10 mM phosphate buffer (pH = 8.0) containing 10 mM NaCl, to a final concentration of 50 μM in the absence or in the presence of compounds **2-9** at 10 μM. Samples were then incubated without stirring at 30 °C for 24 h. To quantify amyloid fibril formation, the thioflavin T fluorescence method was used.[12, 13] After incubation, samples were diluted to a final volume of 2.0 mL with 50 mM glycine-NaOH buffer (pH = 8.5) containing 1.5 □M thioflavin T. A 300-seconds-time scan of fluorescence intensity was carried out (□$_{exc}$ = 446 nm; □$_{em}$ = 490 nm), and values at plateau were averaged after subtracting the background fluorescence of 1.5 □M thioflavin T solution.

***Cellular assays procedures***

Preparation of chicken telencephalic neurons was performed according to Pettmann et al. 1979[14] with modifications.[15] Embryonic day 8 (E8) embryos from fertilized Lohman Brown hybrid chicken eggs were isolated, and telencephalic neurons were mechanically dissociated using a sterile nylon sieve with pore size 100 μM (BD Falcon). Neurons were maintained in Dulbecco's Modified Eagles Medium containing 4.5 g glucose/L, 5% Nu Serum, 0.01% gentamicin, and 2 mM L-glutamine.

For the Aβ secretion assay, chicken neurons were plated on poly-D-lysine pre-coated 24-well plates (BD Biocoat) at a cell density of $1.8 \times 10^6$ neurons per 24 well. After 48 h, culture medium was exchanged, and neurons were incubated for 24 h with different concentrations of compounds in a total volume of 300 μL culture medium. For the MTT viability assay, neurons were plated on poly-D-lysine precoated 96-well plates (BD Biocoat) at a cell density of $3 \times 10^5$ neurons per 96 well in a total volume of 160 μL culture medium. All cultures were kept at 37 °C and 5% $CO_2$. Viability of cultures using the MTT assay was performed as previously described.[16]

**Evaluation**

Determination of Aß species

After treatment, cell supernatants were snap frozen at −80 °C and defrosted prior to Aβ measurement. At least three separate wells were analyzed per experiment for each condition. Cell supernatants were examined for $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ content with a commercially available Aβ-Triplex kit from Mesoscale Discovery (MSD). Conditioned media of primary chicken telencephalic cultures were measured undiluted. For capture, $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ specific antibodies binding the C-terminus of the corresponding Aβ peptide (provided by MSD) were used. Bound Aβ peptides were detected with a monoclonal 6E10 antibody and quantified by electrochemiluminescence. Aβ levels in cell supernatants were evaluated in comparison to an Aβ peptide standard in picogram per milliliter. Vehicle treated control cultures were set as 100%. All data of compound-treated cultures are displayed in relation to vehicle-treated controls as mean ± standard error of mean.

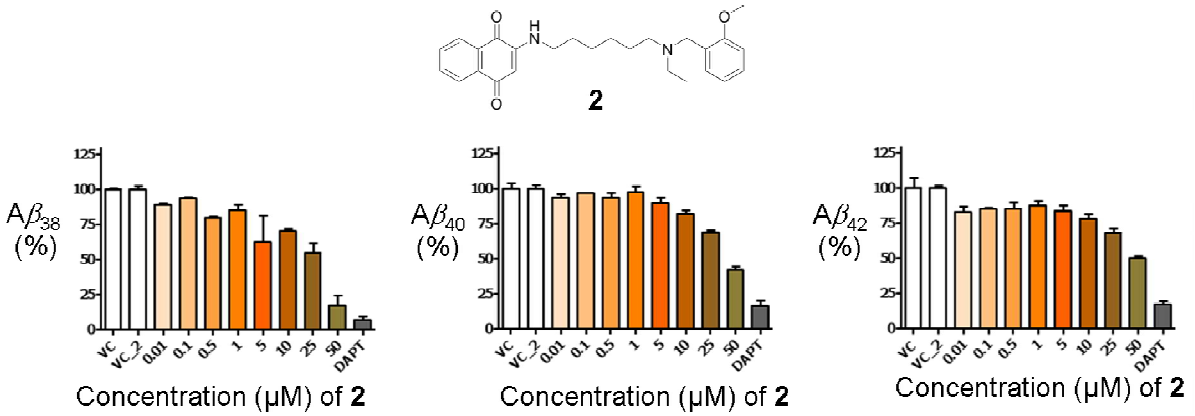



**Figure 1SI. Secretion of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ after treatment with 2. 2** reduces Aβ secretion at μM concentrations. Values represent the mean and S.E.M. of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ secretion in percent (n=3 per experiment) for each concentration and for vehicle controls. Vehicle controls were set as 100% (VC, VC_2). The gamma-secretase inhibitor DAPT was used for reference. Statistical analysis was performed using one-way ANOVA analysis followed by Bonferroni's Multiple Comparison Test.

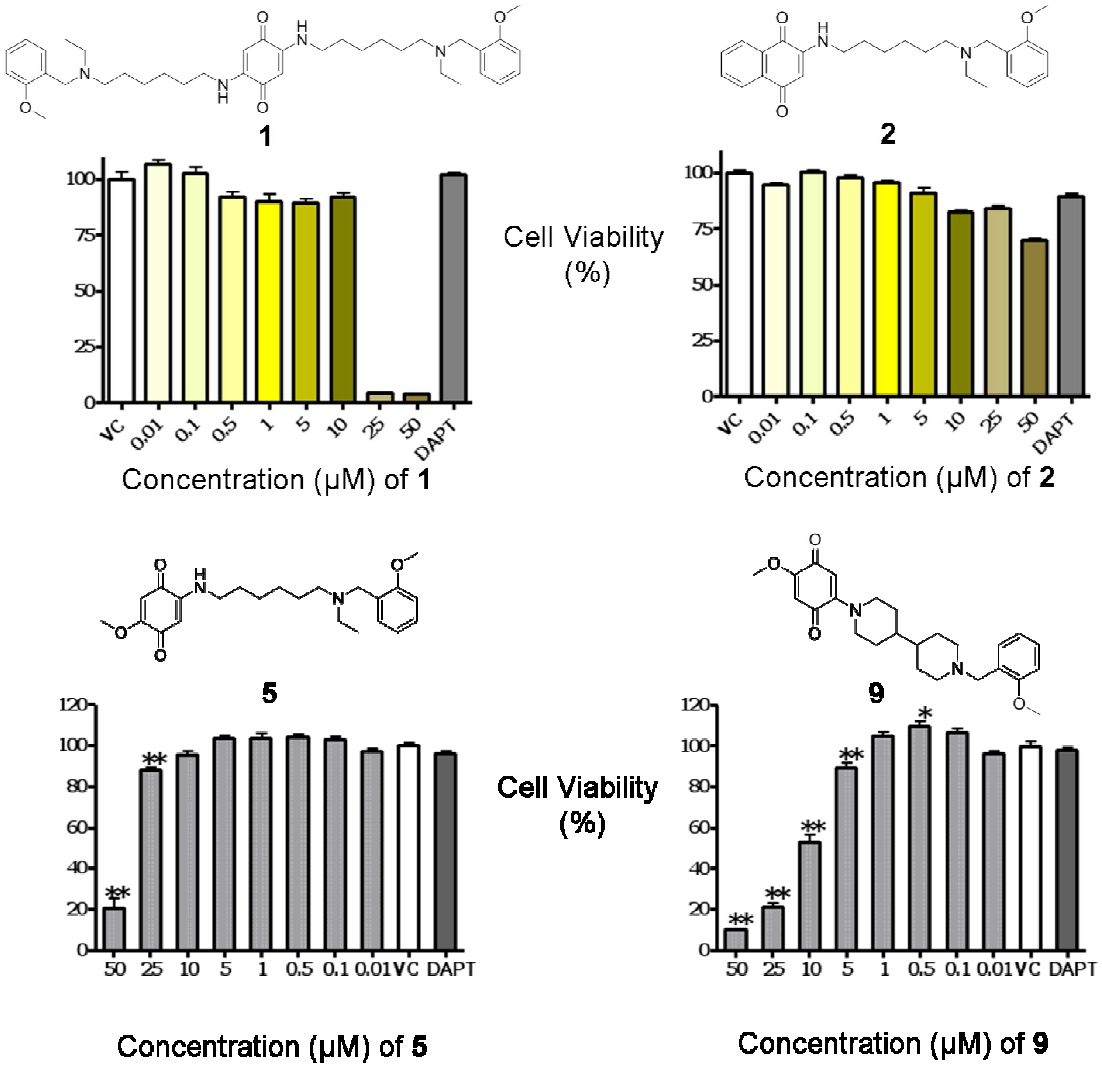



**Figure 2SI.** Neurotoxicity of compounds **1**, **2**, **5** and **9** after 24 h of treatment. Values represent the mean neuronal viability in percent and S.E.M. (n = 6 per experiment) for each concentration and for controls. The vehicle control group (VC) was set as 100%. Statistical analysis was performed using one-way ANOVA analysis followed by Bonferroni's Multiple Comparison Test.

**Statistics**

Descriptive statistical analysis was performed. Data will be represented as mean ± S.D. or (S.E.M.). To determine statistical significant differences between treatment groups, one-way ANOVA analyses followed by Bonferroni's Multiple Comparison Tests were performed.

**Computational Methods**

The docking simulations were carried out by means of ICM 3.7.

AChE was modelled starting from the crystallographic structure of human AChE in complex with fasciculin (PDBid: 1B41)[17]. Fasciculin and other non protein molecules were deleted and only the enzyme chain was retained. Hydrogen atoms and missing heavy atoms were added. Zero occupancy side chains, polar hydrogen atoms, and the positions of asparagine and glutamine side chain amidic groups were optimized and assigned the lowest energy conformation. After optimization, histidines were assigned the tautomerization state which improved the hydrogen bonding pattern.

The residues defining the boundaries of the binding box were assumed to be known and directly derived from the bound poses of the two inhibitors: propidium (in complex with mouse AChE; PDBid: 1N5R) and donepezil (in complex with torpedo AChE; PDBid: 1EVE).[18, 19]

Likewise, BACE-1 was modelled starting from the crystallographic structure of the human enzyme in complex with a peptide-mimetic inhibitor (PDBid: 2QZL).[20]

Ligands were built defining the right bond orders, hydrogen bonds, and protonation states. Each ligand was assigned the MMFF force field[21] atom types and charges.

The docking engine employed was the Biased Probability Monte Carlo (BPMC) stochastic optimizer as implemented in ICM.[22-24] The ligand binding site at the receptor was represented by precalculated 0.5 Å spacing potential grid maps, representing van der Waals potentials for hydrogen and heavy probes, electrostatics, hydrophobicity, and hydrogen bonding. The molecular conformation was described by means of internal coordinate variables. The adopted force field was a modified version of the ECEPP/3 force field.[25] The binding energy was assessed by means of the standard ICM empirical scoring function.[26]

**Table 1SI.**

| Cmpd | MW (Da) | cLogP | TPSA (Å) | Solubility | N° violations of Lipinski's Rule of Five |
|---|---|---|---|---|---|
| **1** | 634.4 | 6.1 | 71.6 | -6.8 | 2 |
| **2** | 421.2 | 4.7 | 48.9 | -6.3 | - |
| **3** | 422.2 | 3.6 | 58.8 | -5.1 | - |
| **4** | 399.3 | 3.9 | 49.1 | -4.2 | - |
| **5** | 401.2 | 3.2 | 56.5 | -3.6 | - |
| **6** | 378.2 | 4.9 | 54.8 | -5.8 | - |
| **7** | 273.2 | 3.5 | 59.0 | -4.2 | - |
| **8** | 461.3 | 3.4 | 51.7 | -4.4 | - |
| **9** | 425.2 | 2.8 | 48.4 | -2.0 | - |

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