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Supporting Information

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A New Small Molecule Targeting the Multifactorial Nature of Alzheimer's Disease

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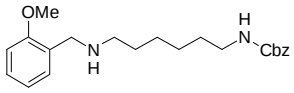
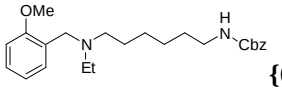
1. Synthesis and characterization of compounds 1-4

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. Electron impact (EI) mass, and HRMS spectra were recorded on VG 7070E and MAT95XP Finnigan apparatus, respectively. ^{1}H NMR were recorded on Varian VXR 300 instrument. 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). Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040-0.063 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.

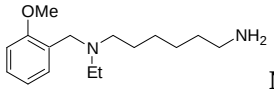
Purity determination. HPLC–ESI-MS analysis was performed under reversed-phase conditions on a Phenomenex phenyl-hexyl (150x3.0 mm I.D.) column, using as the mobile phase a binary mixture of triethylammonium acetate (0.02 M; pH 4.0) buffer/acetonitrile 71/29 (v/v) at a flow rate of 0.4 mL/min. A loop valve of 20 µL volume was used. The liquid chromatograph was by Jasco Corporation (Tokyo, Japan), model PU-1585, interfaced by means of a T split valve with the LCQ Duo Mass Spectrometer (Thermo Finnigan, San Jose, CA, USA). The system was equipped with heated capillary interface and electrospray ionization (ESI) source and operated with both Ion Trap analyzer and Jasco UV-875 detector (wavelength 340 and 220 nm). ESI system employed a 4.5 kV (positive polarity) spray voltage, heated capillary temperature and voltage of 220 °C and 43.93 V, respectively. The sheath gas and the auxiliary gas (nitrogen) flow rate were at 0.75 and 1.2 L/min, respectively. Electrospray ionization was optimized using galantamine as reference compound. The mass chromatograms were acquired in total ion current (TIC) modality from 100 to 400 *m*/*z*, in MS/MS mode (relative collision energy 25%) on the ESI generated ions at *m*/*z* 302 for haemanthamine.
Under the above conditions all samples of **4** resulted to have a purity higher than 98%.



[6-(2-Methoxybenzyl)amine-hexyl]-carbamic acid benzyl ester (1). A solution of (6-amino-hexyl)-carbamic acid benzyl ester (2.50 g; 10 mmol) and 2-methoxybenzaldehyde (1.50 g; 11 mmol) in ethanol (70 mL) containing molecular sieves (4 Å) was stirred at room temperature for 20 min, then $NaBH_4$ (0.4 g; 10 mmol) was added and stirring was continued for further 6 h. The mixture was made cautiously acidic with 2N HCl. The sieves were filtered off and the solvents were evaporated; the residue was taken up with H_2O and extracted with diethyl ether (3 x 20 mL). The aqueous phase was made basic with aqueous 40% NaOH and extracted with $CHCl_3$ (4 x 20 mL). Removal of the dried solvent gave 3.0 g (81% yield) of the title compound as a transparent oil; ^{1}H NMR (free base; 300 MHz; $CDCl_3$) d: 1.22-1.38 (m, 4H, C*H*$_2$ chain), 1.41-1.57 (m, 4H, C*H*$_2$ chain), 1.65 (br s, 1H exchangeable with D_2O, CH_2NHCH_2), 2,58 (t, J = 6.6 Hz, 2H, C*H*$_2$NHCH$_2$Ph), 3.18 (q, J = 6.3 Hz, 2H, C*H*$_2$NHCbz), 3.77 (s, 2H, NHC*H*$_2$Ph), 3.84 (s, 3H, OC*H*$_3$), 4.76 (br s, 1H exchangeable with D_2O, N*H*Cbz), 5.09 (s, 2H, COOC*H*$_2$), 6.82-6.93 (m, 2H, Ar-*H*), 7.20-7. 40 (m, 7H, Ar-*H*).

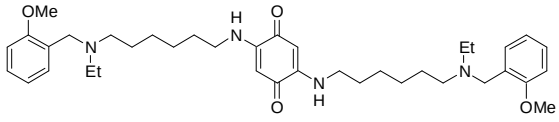


{6-[Ethyl-(2-methoxybenzyl)-amino]-hexyl}-carbamic acid benzyl ester (2). A solution of **1** (2.91 g; 7.85 mmol) and diethyl sulphate (1.24 mL; 9.5 mmol) in toluene (120 mL) was refluxed for 24 h. After cooling to room temperature, the mixture was shaken with aqueous 40% NaOH (50 mL). The organic phase was separated, dried and evaporated to give a crude material which was purified by flash chromatography. Eluting with CH_2Cl_2/petroleum ether/MeOH/aqueous 33% ammonia (4.6:5.0:0.4:0.04) afforded 2.4 g (76% yield) of the title compound as a transparent oil; ^{1}H NMR (free base; 300 MHz; $CDC1_3$) d: 1.05 (t, J = 6.9 Hz, 3H, C*H*$_3$CH$_2$), 1.21-1.32 (m, 4H, C*H*$_2$ chain), 1.40-1.57 (m, 4H, C*H*$_2$ chain), 2.41-2.59 (m, 4H, NC*H*$_2$CH$_2$ + NC*H*$_2$CH$_3$), 3.16 (q, J = 6.3 Hz, 2H, C*H*$_2$NHCbz), 3.60 (s, 2H, NHC*H*$_2$Ph), 3.80 (s, 3H, OC*H*$_3$), 4.73 (br s, 1H exchangeable with D_2O, N*H*Cbz), 5.08 (s, 2H, COOC*H*$_2$), 6.82-6.94 (m, 2H, Ar-*H*), 7.18-7.42 (m, 7H, Ar-*H*).



N^1-ethyl-N^1-(2-methoxybenzyl)-hexane-1,6-diamine (3). To a cold solution of **2** (2.31 g; 6.01 mmol) in glacial CH_3COOH (15 mL), a 33% solution of HBr in CH_3COOH (5 mL) was cautiously added. The resulting mixture was stirred for 2 h at room temperature, then the hydrobromide salt was precipitated with diethyl ether (100 mL). The collected white solid was dissolved in H_2O, made basic with 2N NaOH and extracted with $CHC1_3$ (5 x

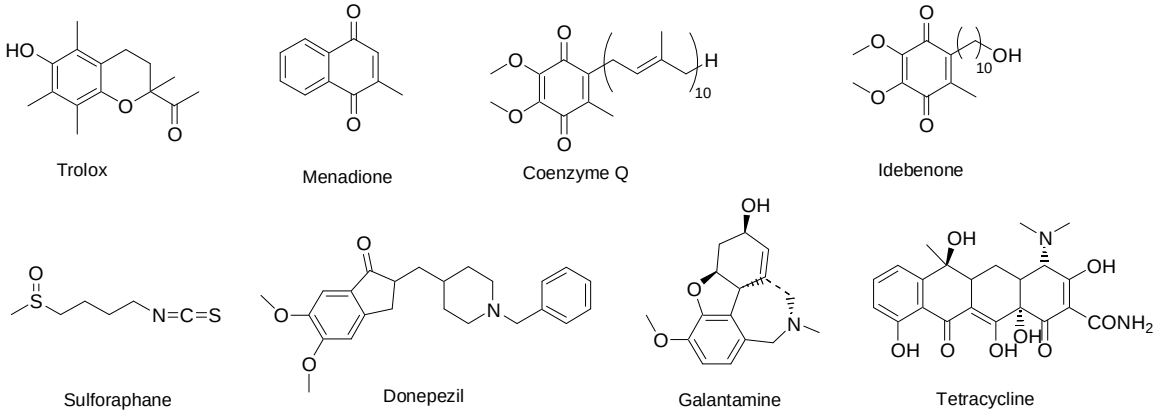
20 mL). Removal of the dried solvent gave 1.55 g (98% yield) of the title compound as a transparent oil; ^{1}H NMR (free base; 300 MHz; $CDC1_3$) d: 1.04 (t, J = 6.9 Hz, 3H, C*H*$_3$CH$_2$), 1.12-1.48 (m, 8H + 2H exchangeable with D_2O, C*H*$_2$ chain + N*H*$_2$), 2.41-2.53 (m, 4H, NC*H*$_2$CH$_2$ + NC*H*$_2$CH$_3$), 2.65 (t, J = 6.9 Hz , 2H, C*H*$_2$NH$_2$), 3.57 (s, 2H, NHC*H*$_2$Ph), 3.81 (s, 3H, OC*H*$_3$), 6.82-6.94 (m, 2H, Ar-*H*), 7.16-7.42 (m, 2H, Ar-*H*).



2,5-Bis-{6-[ethyl-(2-methoxybenzyl)-amino]-hexylamino}-[1,4]benzoquinone (memoquin, 4). A solution of p-benzoquinone (0.2 g; 1.84 mmol) and **3** (1.05 g; 4 mmol) in MeOH (70 mL) was stirred in the dark at room temperature for 6 h, then the solvent was evaporated to give a crude material which was purified by flash chromatography. Eluting with CH_2C1_2/MeOH/aqueous 33% ammonia (9.75:0.25:0.025) afforded 0.2 g (17% yield) of the title compound as a red solid; m.p. 45 °C; ^{1}H NMR (free base; 300 MHz; $CDC1_3$) d: 1.04 (t, J = 6.9 Hz, 6H, C*H*$_3$CH$_2$), 1.14-1.38 (m, 8H, C*H*$_2$ chain), 1.43-1.53 (m, 4H, C*H*$_2$ chain), 1.57-1.65 (m, 4H, C*H*$_2$ chain), 2.41-2.56 (m, 8H, NC*H*$_2$CH$_2$ + NC*H*$_2$CH$_3$), 3.10 (q, J = 6.6 Hz, 4H, CONHC*H*$_2$), 3.57 (s, 4H, NHC*H*$_2$Ph), 3.81 (s, 6H, OC*H*$_3$), 5.28 (s, 2H, C*H*CO), 6.59 (br t, 2H exchangeable with D_2O, N*H*), 6.83-6.95 (m, 4H, Ar-*H*), 7.21 (t, J = 7.5 Hz, 2H, Ar-*H*), 7.39 (d, J = 6.9 Hz, 2H, Ar-*H*). EI-MS (70 eV): *m*/*z* (%): 632 (10) [M^+], 121 (100) [$C_8H_9O^+$]. HRMS: calculated for $C_{38}H_{56}N_4O_4$: 632.43016. Found: 632.42970. Purity > 98% (see above).

2. Biology

Chemical structures of the reference compounds used for the determination of the biological activity of 4



H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Stat-Val-Ala-Glu-Phe-OH [Stat = (3S,4S)-Statine]

Statine-derivative

Determination of antioxidant activity by Randox Total Antioxidant Status assay kit. The ability of **4** to neutralize free radicals was assayed by using Randox Total Antioxidant Status assay kit (Randox Laboratories ltd, UK) using Trolox as standard and following the manufacturer's protocol. This colorimetric method is based on reactivity of the peroxidase compound metmyoglobin (6.1 μM) ($HXFe^{3+}$) that, in the presence of hydrogen peroxide (250 μM) in buffer (pH 7.4), allows the formation of oxygen radicals.[1] Moreover, metmyoglobin itself is transformed to ferryl myoglobin ($^?$X-[Fe^{4+}=O]). Ferryl myoglobin abtracts an electron to a cation (ABTS®) (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) (610 μM), transforming back to metmyoglobin and converting ABTS® in a colored radical, quantifiable at 600 nm.[1] The capture of free oxygen radicals by antioxidants reduces the formation of the colored species and the correspondent absorbance. The total amount of oxidant was represented by metmyoglobin and ABTS® incubated at 37 °C in phosphate buffer (80 mM, pH 7.4). The absorbance was read at t_0 and exactly after 3 min, and the ΔAbs was determined. The percentage of inhibition was calculated on the basis of the equation: % inhibition = [($\Delta Abs_{blank} - \Delta Abs_{test}$)/$\Delta Abs_{blank}$) x 100] were ΔAbs_{test} and ΔAbs_{blank} are the differences in absorbance recorded in presence and in absence of the tested compound, respectively.

Substrate specificity for NQO1. **4** was tested with respect to its ability to accept electrons from NADH via human NQO1 (Sigma), by following the absorbance change of cytochrome *c* as secondary acceptor. Menadione was used as reference compound. Briefly, each reaction consisted of NADH, cytochrome *c*, NQO1, and test compounds in a final volume of tris-HCl buffer, containing bovine serum albumin. Reactions were started by the addition of NADH and the reduction of cytochrome *c* was monitored at 550-540 nm. Results were expressed in terms of apparent V_{max} and K_m for two independent experiments.

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.[2] 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, Salizburg, 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 on SH-SY5Y cells. 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.[3] 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.

Determination of inhibitory effect on AChE activity. The capacity of **4** to inhibit AChE activity was assessed using the Ellman's method.[4] Initial rate assays were performed at 37 °C with a Jasco V-530 double beam Spectrophotometer: the rate of increase in the absorbance at 412 nm was followed for 5 min. AChE stock solution was prepared by dissolving human recombinant AChE (E.C.3.1.1.7) lyophilized powder (Sigma, Italy) in 0.1 M phosphate buffer (pH 8.0) containing Triton X-100 0.1%. Stock solutions of **4** (1 mM) were prepared in methanol and diluted in bidistilled water. Five increasing concentrations of the inhibitor were used, able to give an inhibition of the enzymatic activity in the range of 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, and 550 μM of substrate (acetylthiocholine 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 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 **4** (range 0 – 3.70 nM) in a weighted analysis and K_i was determined as the intersect on the negative x-axis.
Reciprocal plots involving **4** inhibition (**Figure S1**) show both increasing slopes (decreased V_{max} at increasing inhibitor concentrations) and increasing intercepts (higher K_m) with higher inhibitor concentration. This pattern indicates mixed-type inhibition.

Determination of inhibitory effect on Aβ(1-40) aggregation induced by human AChE. Aliquots of 2 μL of Aβ(1-40) (Bachem AG, Switzerland) peptide, lyophilized from a 2 mg/ml 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solution and dissolved in DMSO at a final concentration of 230 μM, were incubated for 24 h at room temperature in 0.215 M Na phosphate buffer (pH 8.0). For coincubation experiments, aliquots of AChE (2.30 μM, Aβ/AChE ratio 100:1) and AChE in the presence of **4** (concentration range 10-250 μM) were added.
Blanks containing Aβ, AChE, Aβ plus **4**, and AChE plus **4** in 0.215 M Na phosphate buffer (pH 8.0) were prepared. The final volume of each vial was 20 μL. To quantify amyloid fibril formation, the thioflavin T fluorescence method was used.[5-8] Thioflavin T binds to amyloid fibrils, giving rise to an intense specific emission band at 490 nm in its fluorescent emission spectrum. Therefore, after incubation, samples were diluted to a final volume of 2 mL with 50 mM

glycine-NaOH buffer (pH 8.5) containing 1.5 μM thioflavin T. A 300-seconds-time scan of fluorescence intensity was carried out (λ_{exc} = 446 nm; λ_{em} = 490 nm), and values at plateau were averaged after subtracting the background fluorescence of 1.5 μM thioflavin T solution. The percent inhibition of the AChE induced aggregation due to the presence of **4** was calculated by the following expression: 100-(IF_i/IF_o x 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.

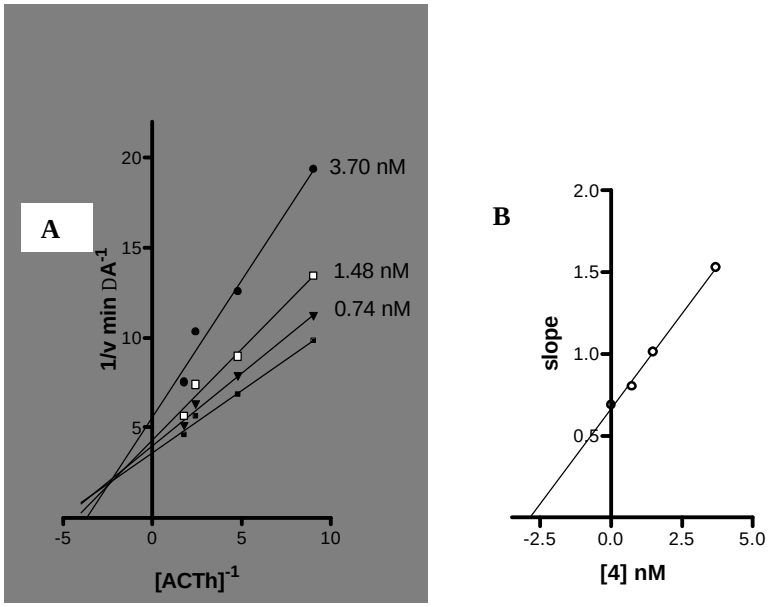


Figure S1. Inhibition of AChE activity by memoquin (**4**). (A) Steady-state inhibition of AChE hydrolysis of acetylthiocholine (ACTh) by **4**. Lineweaver-Burk reciprocal plots of initial velocity and substrate concentrations are presented. Lines were derived from a weighted least-squares analysis of the data points. (B) Plot of slopes for **4** at different concentrations, as derived from Lineweaver-Burk plot, showing a K_i of 2.60 ± 0.48 nM.

Determination of inhibitory effect on Ab(1-42) self-aggregation. In order to investigate the Aβ(1-42) self-aggregation, a Thioflavin-T based fluorometric assay was performed. HFIP pretreated Aβ(1-42) samples (Bachem AG, Switzerland) were resolubilized with a CH_3CN/Na_2CO_3/NaOH (48.4/48.4/3.2) mixture in order to have a stable stock solution ([Aβ 1-42] = 500 μM). Experiments were performed by incubating the peptide in 10 mM phosphate buffer (pH 8.0) containing 10 mM NaCl, at 30 °C for 24 h (final Aβ concentration 50 μM) with and without **4**. Tested inhibitor concentrations ranged from 1.0 to 50.0 μM (Aβ/**4** from 1/50 to 1/1). Blanks containing **4** were also prepared and tested. To quantify amyloid fibril formation, the thioflavin T fluorescence method was used.[5, 6] 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 μM thioflavin T. A 300-seconds-time scan of fluorescence intensity was carried out (λexc = 446 nm; λem = 490 nm), and values at plateau were averaged after subtracting the background fluorescence of 1.5 μM thioflavin T solution.

Determination of inhibitory effect on BACE-1 activity. BACE-1 (Invitrogen, Italy) activity was measured with a fluorescence resonance energy transfer assay[9] by using Fluoroskan Ascent FL (Labsystems, Italy), a fluorescent 96-well multiplate reader. The peptide substrate for the analysis mimics the APP protein, which is the BACE-1 natural substrate. This synthetic substrate (Rh- EVNLDAEFK-Quencher) contains two groups, a fluorescent donor (a rhodamine derivative) and a quenching acceptor (Invitrogen, Italy). The weakly fluorescent substrate becomes highly fluorescent upon enzymatic cleavage; the increase in fluorescence is linearly related to the rate of proteolysis.
In the optimized conditions of analysis (incubation time: 60 minutes, temperature: 37 °C, λexc = 540 nm, λem = 590 nm, Substrate: 250 nM, Enzyme 1 U/mL), the assay allowed us to measure BACE-1 activity and its inhibition by evaluating the loss in fluorescence. The fluorescence intensities with and without inhibitor were compared and the % inhibition due to the presence of test compounds was calculated. The % inhibition due to the presence of increasing test compound concentration was calculated by the following expression: 100-(IFi/IFo x 100) where IFi and IFo 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 against the logarithm of inhibitor concentration in the assay sample. The linear regression parameters were determined and, where possible, the IC_{50} extrapolated (GraphPad Prism 3.0, GraphPad Software Inc.). To demonstrate inhibition of BACE-1 activity, a statine-derived inhibitor (Calbiochem, San Diego CA, U.S.A.) was serially diluted into the reactions' wells (IC_{50} = 18 ±2 nM).

Animals and treatments. All mice used in these experiments were housed at constant room temperature (22 ± 1 °C) and relative humidity (60 ± 1%) under a 12 h light/dark cycle. Food and water were provided *ad libitum*. Effects of treatment on body weight and mortality were recorded. All experiments were conducted according to the guidelines of the European Animal Health and Welfare Act. AD11 anti-NGF mice were produced as described before.[10] **4** (7 mg/kg/day) was administered intraperitoneally for 15 days to 2-, 6- and 15-month-old AD11 mice (n = 4 per group of treatment). To assess the effects on behavioural impairment, 6- and 12-month-old AD11 mice received Memoquin per os (15 mg/kg/day).

Neurohistological analysis and biochemistry. At the end of the treatment, animals were anesthetized with 8 μl/g of 10.5% chloral hydrate solution. Brains were fixed in 4% paraformaldehyde/phosphate buffered saline and processed for immunohistochemistry as described.[11] The following primary antibodies were used: anti-choline acetyltransferase (ChAT, 1:500, Chemicon, Temecula, CA), anti-phosphorylated tau (clone AT8, 1:10, Innogenetics, Gent, Belgium), and anti-β-amyloid (polyclonal R3660, kindly provided by Gennaro Schettini and Claudio Russo, University of Genoa, Italy). Quantitative neurostereology was performed as described before.[12] Statistical analysis was performed using an ANOVA one way t test. Biochemical analysis on P301L brain extracts was performed according to Sahara et al.[13]

3. Computational studies.

Docking and molecular dynamics simulations. Docking experiments were carried out with the AutoDock 3.0.5 software.[14] 100 docking runs were carried out, and a minimum energy conformation was then submitted to molecular dynamics (MD) simulations performed with the AMBER 8 package (University of California, San Francisco, 2004). MD studies were carried out on an NPT ensemble (constant temperature of 298 K and pressure of 1 atm), in explicit solvent, and employing periodic boundary conditions. van der Waals and short range electrostatic interactions were estimated within a 10 Å cutoff, whereas the long range electrostatic interactions were assessed by using the particle mesh Ewald method. The MD simulations were carried out for 5 ns with a time step of 1.5 fs. The conformation reported in figure 1 was selected within a stable simulation interval, and its geometry was energetically minimized with the steepest descent and the conjugate gradient algorithms. All the calculations were performed on a Linux cluster employing an open-Mosics® architecture.

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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