

## Supporting Information

### Novel Curcumin-Diethyl Fumarate Hybrid as a Dualistic GSK-3 $\beta$ Inhibitor/Nrf2 Inducer for the Treatment of Parkinson's Disease

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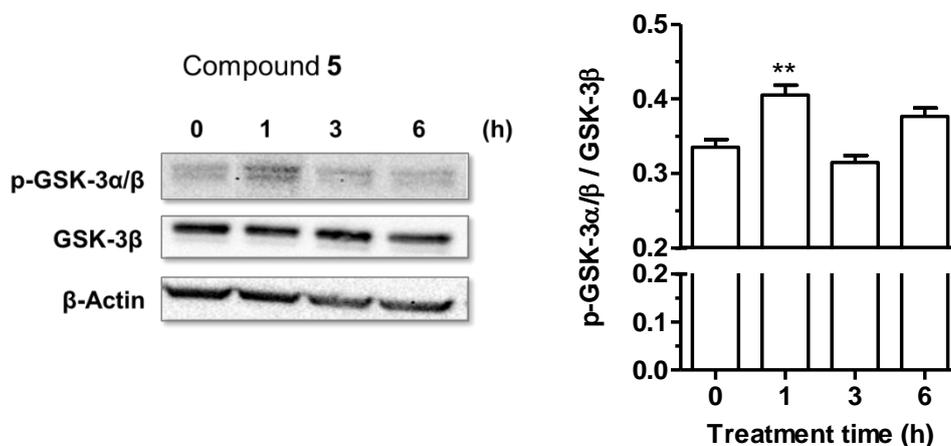
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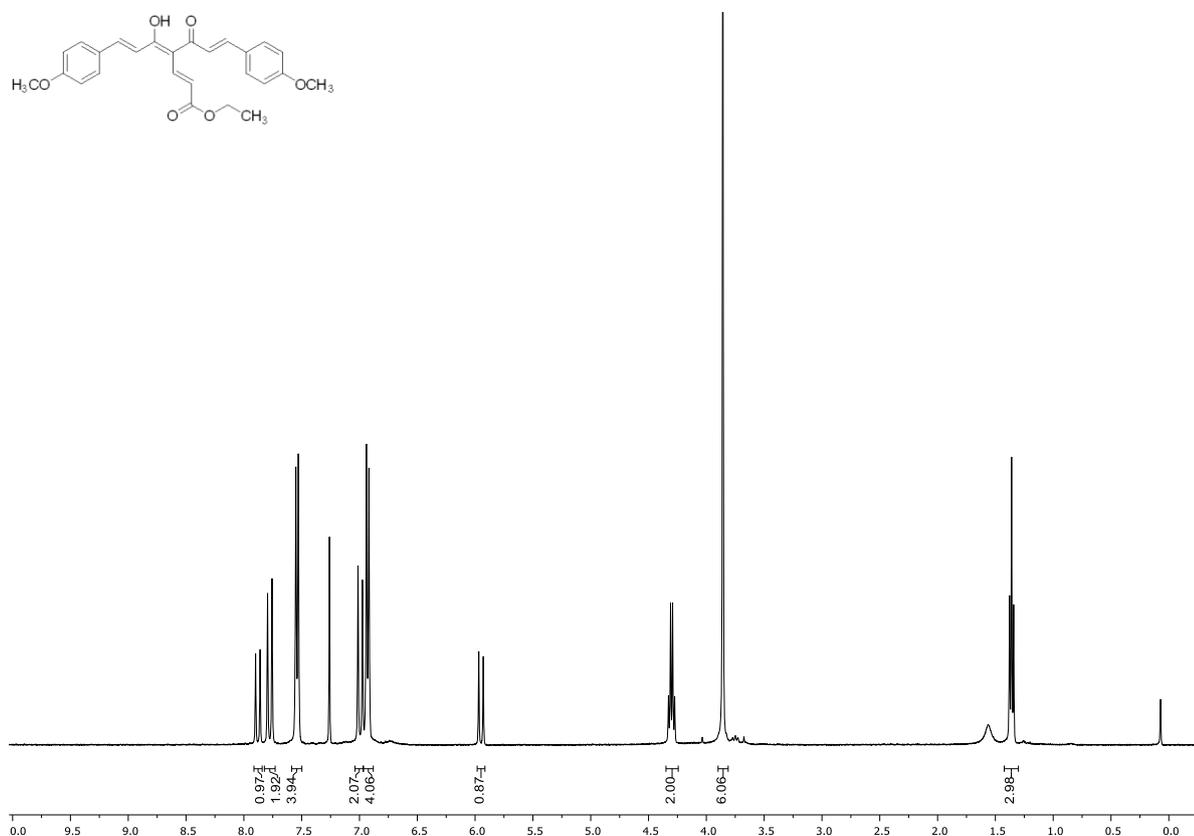
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## GSK-3 $\beta$ activity in SH-SY5Y cells



**Figure S1. Inhibition of GSK-3 $\beta$  activity in SH-SY5Y cells.** Cells were incubated with compound 5 (5  $\mu$ M) for different times (1, 3 and 6 h). At the end of incubation, the phosphorylation of GSK-3 $\alpha/\beta$  (Ser21/9) was determined by Western blotting, as described in the materials and methods section. Data are expressed as the ratio between phospho-GSK-3 $\alpha/\beta$  and GSK-3 $\beta$  levels normalised against  $\beta$ -Actin and reported as mean  $\pm$  SEM of at least three independent experiments (\*\*  $p < 0.01$  versus untreated cells at one-way ANOVA with Dunnett post hoc test).



**Figure S2.** <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz) of compound 4.

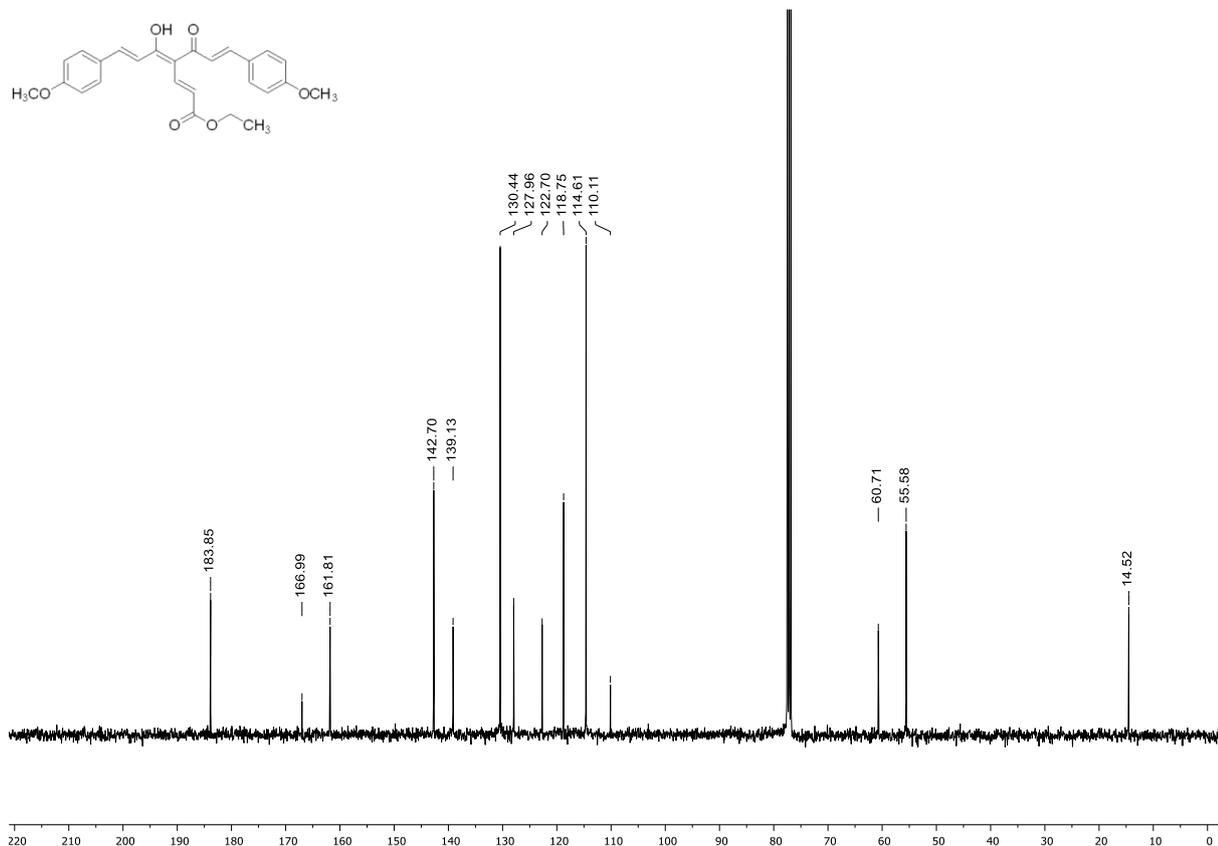


Figure S3.  $^{13}\text{C-NMR}$  (CDCl<sub>3</sub>, 101 MHz) of compound 4.

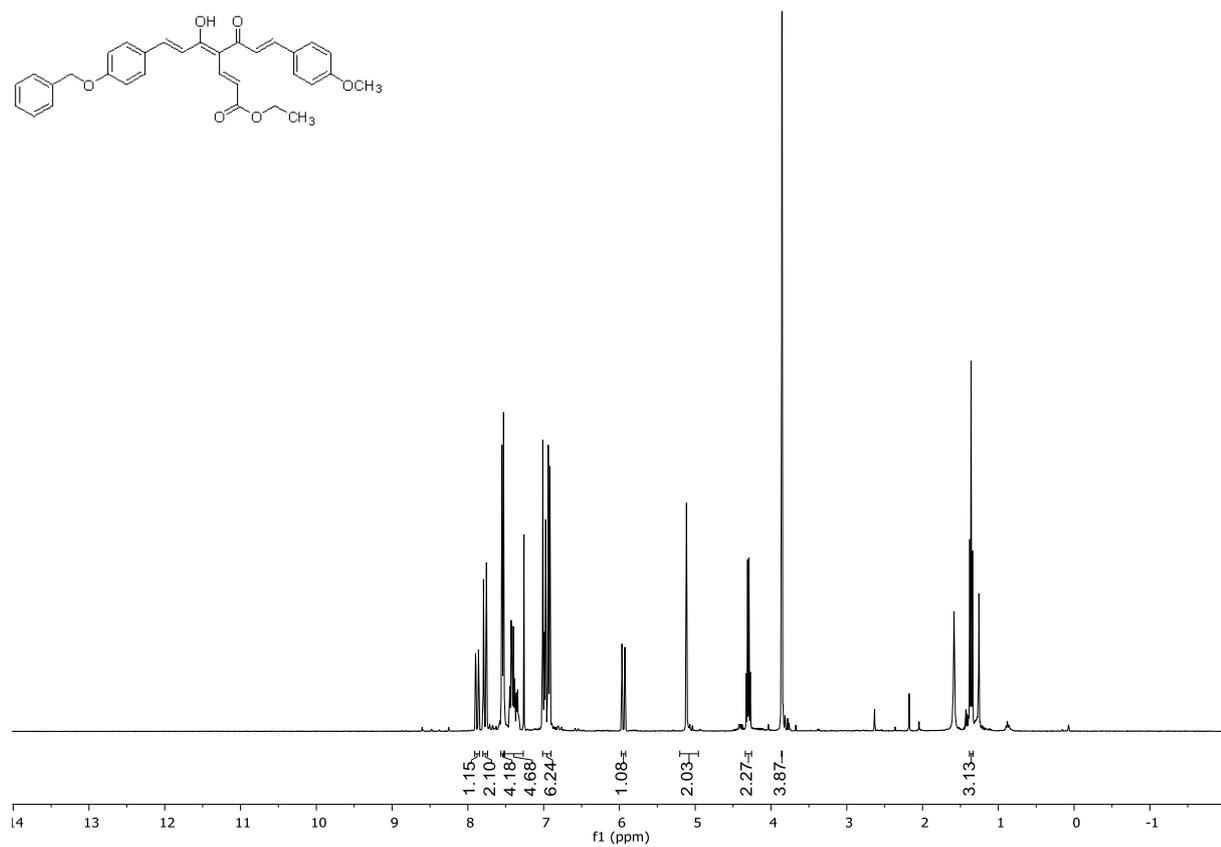


Figure S4.  $^1\text{H-NMR}$  (CDCl<sub>3</sub>, 400 MHz) of compound 5.

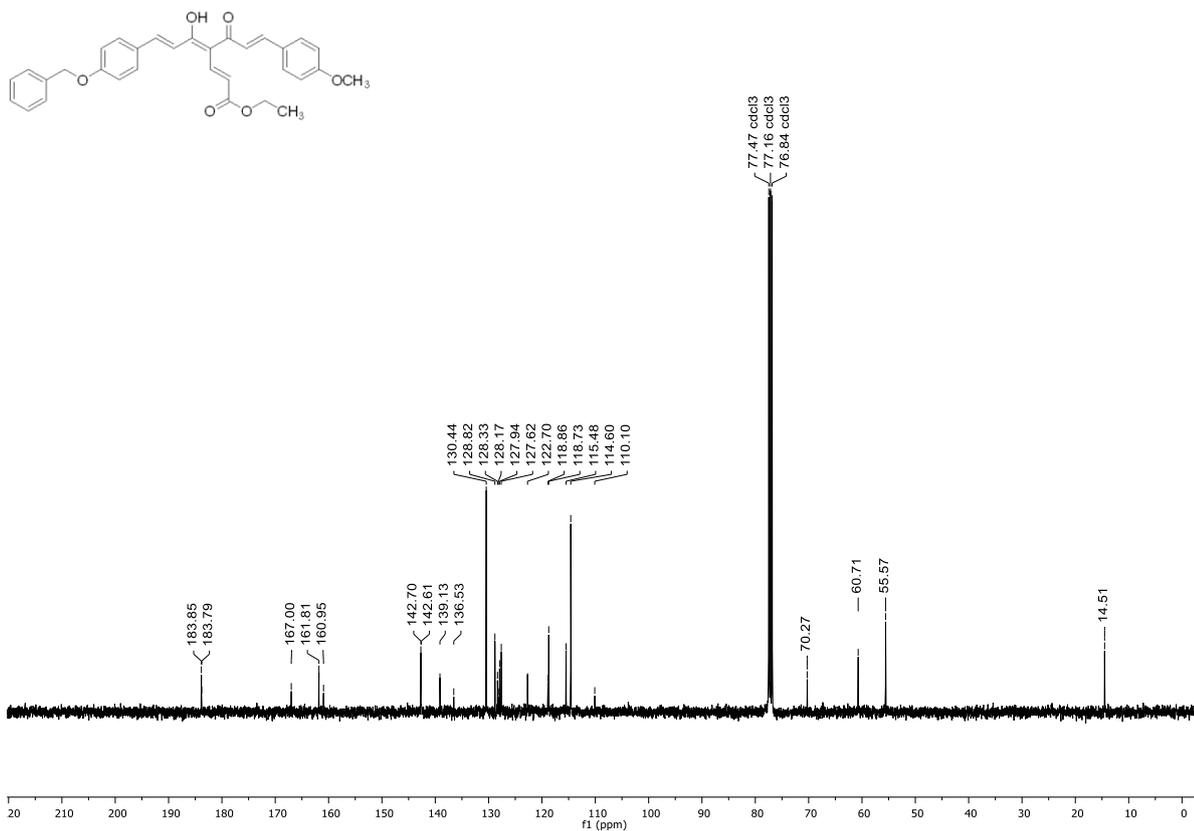


Figure S5.  $^{13}\text{C-NMR}$  (CDCl<sub>3</sub>, 101 MHz) of compound 5.

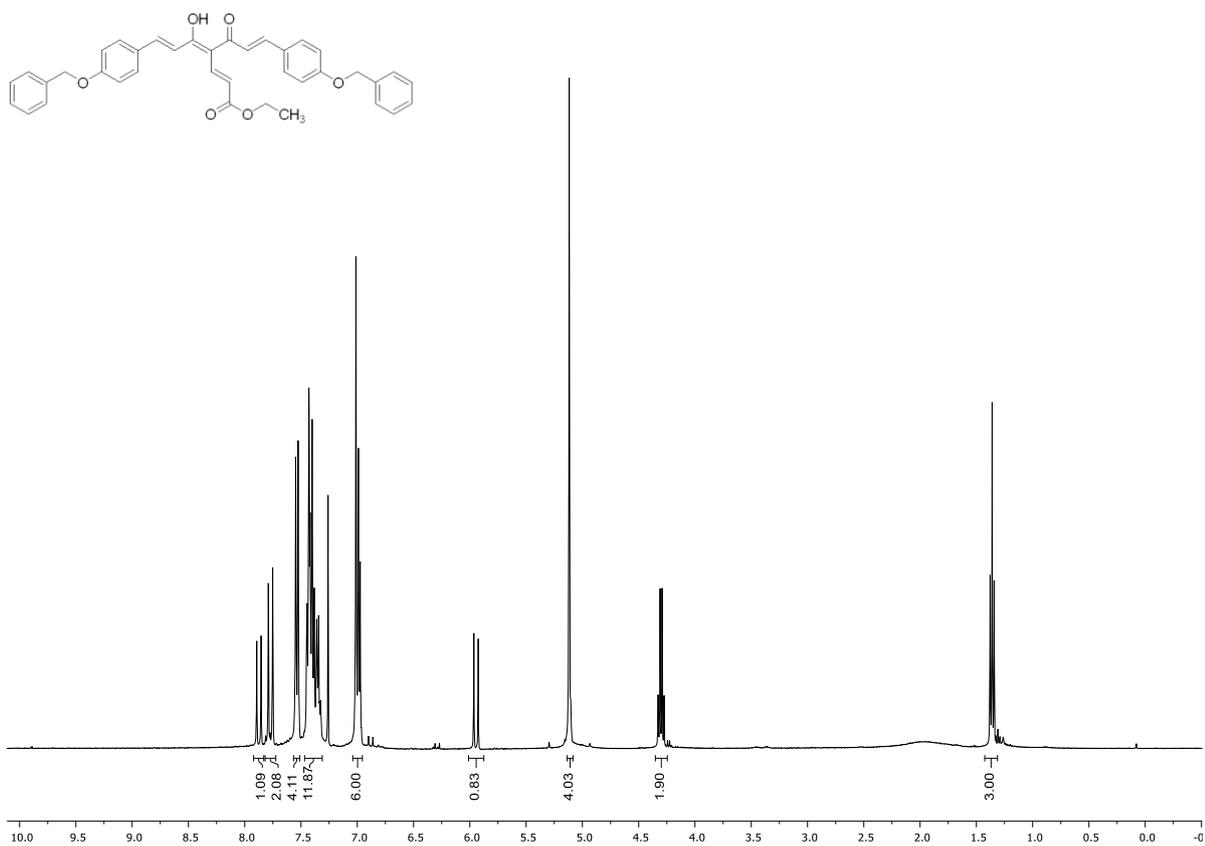
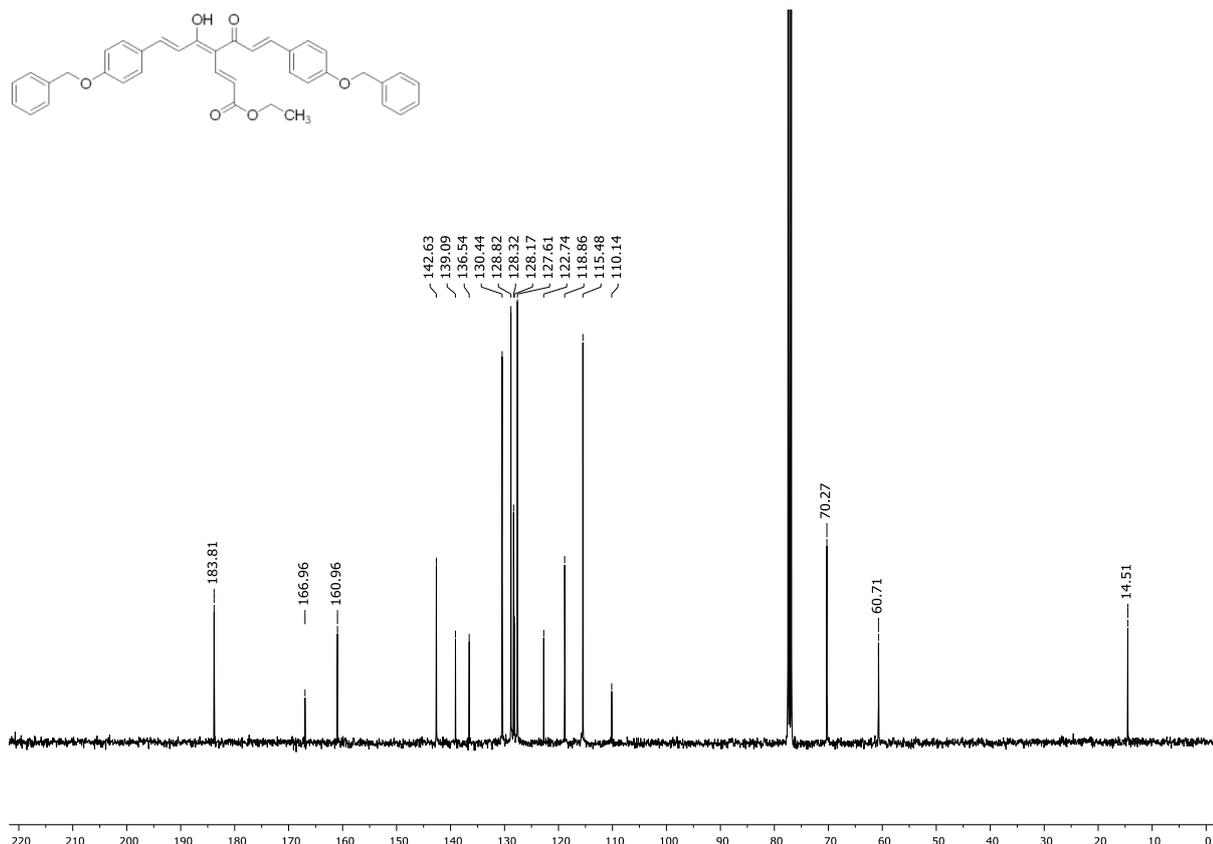


Figure S6.  $^1\text{H-NMR}$  (CDCl<sub>3</sub>, 400 MHz) of compound 6.



**Figure S7.** <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 101 MHz) of compound 6.

### Blood-Brain Barrier (BBB) Permeation

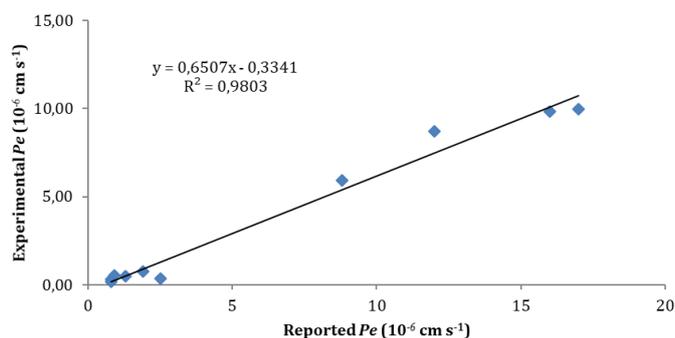
To experimentally validate the PAMPA-BBB assay, *in vitro* permeabilities (*Pe*) values of commercial drugs were determined (Table 1) and then compared with the experimental data of the new compounds, obtained employing this methodology.

**Table S1.** Permeability (*Pe* 10<sup>-6</sup> cm s<sup>-1</sup>) in the PAMPA-BBB assay for 10 commercial drugs (used in the experiment validation) with their predictive penetration in the CNS.<sup>a</sup>

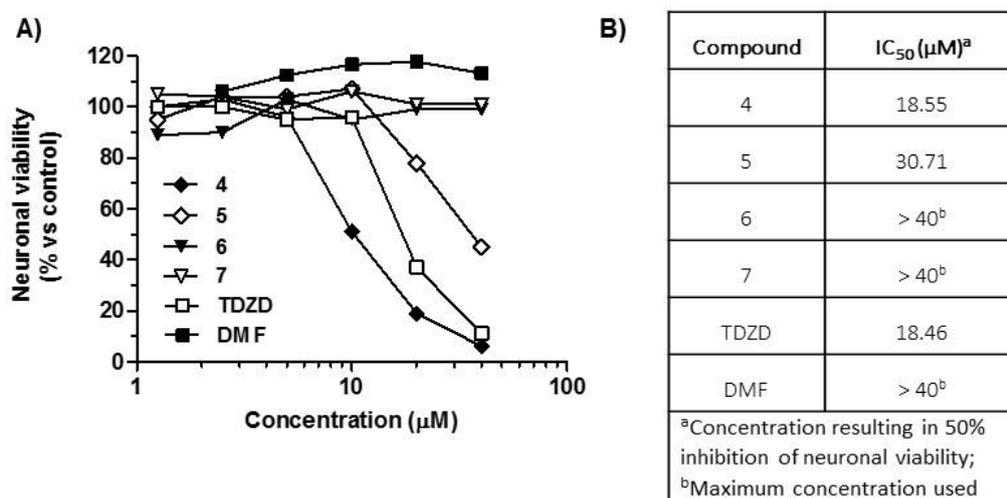
Compound	Bibl. <sup>b</sup>	<i>Pe</i> (10 <sup>-6</sup> cm s <sup>-1</sup> ) <sup>c</sup>		
Atenolol	0.8	0.3	±	0,1
Caffeine	1.3	0.5	±	0,1
Desipramine	12	8,7	±	1,0
Enoxacin	0.9	0.5	±	0,1
Hydrocortisone	1.9	0.8	±	0,6
Ofloxacin	0.8	0.2	±	0,1
Piroxicam	2.5	0.4	±	0,1
Promazine	8.8	5.9	±	0,7
Testosterone	17	10.0	±	1,1
Verapamil	16	9.8	±	0,5

<sup>a</sup>PBS:EtOH (70:30) was used as solvent. <sup>b</sup> See Reference <sup>1</sup>. <sup>c</sup>Datmonga are the mean ± SD of 2 independent experiments.

A good correlation ( $R^2 = 0.9803$ ) between experimental (exptl) and the corresponding values described in literature (bibl) was obtained:  $Pe$  (exptl) =  $0.6507$  (bibl) –  $0.3341$  (Figure S8).



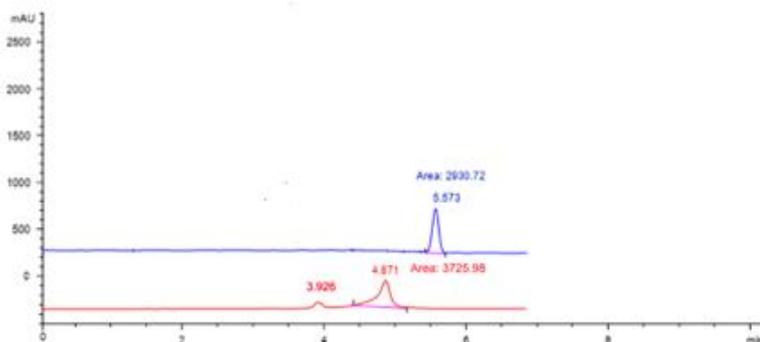
**Figure S8.** Linear correlation among experimental and reported permeability of commercial drugs using the PAMPA-BBB assay.



**Figure S9.** Cytotoxicity of compounds 4-7, TDZD and DMF in SH-SY5Y cells. A) Cells were incubated for 24 h with different concentrations of the studied compounds [1.25 - 40 μM]. At the end of incubation, the neuronal viability was measured using MTT assay, as described in the materials and methods section. Data are expressed as percentage of neuronal viability versus untreated cells and reported as mean of two independent experiments; B) IC<sub>50</sub> values of compounds.

**Table S2.** Primer sequences for quantitative Real-Time PCR.

Gene name	Forward/Reverse	5' to 3' Sequence
NQO1	For	GGGATCCACGGGGACATGA
	Rev	ATTTGAATTCGGGCGTCTGC
B2M	For	CTTCCATTCTGCTGGATGACG
	Rev	GCGGGCATTCTGAAGCTGACAGCA
TBP	For	CACATCACAGCTCCCCACCA
	Rev	TGCACAGGAGCCAAGAGTGAA



**Figure S10.** RP-HPLC copy of compounds **6** (blu) and **7** (red).

## MATERIALS AND METHODS

**GSK-3 $\beta$  inhibition.** Kinase-Glo assays (Promega Biotech Iberica, SL) were performed in assay buffer using black 96-well plates. In a typical assay, 10  $\mu$ l (10  $\mu$ M) of test compound (dissolved in DMSO at 1 mM concentration and diluted in advance in assay buffer to the desired concentration) and 10  $\mu$ l (20 ng) of enzyme were added to each well followed by 20  $\mu$ l of assay buffer containing 50  $\mu$ M substrate and 2  $\mu$ M ATP. The final DMSO concentration in the reaction mixture did not exceed 1 %. After 30-min incubation at 30  $^{\circ}$ C the enzymatic reaction was stopped with 40  $\mu$ l of Kinase-Glo reagent. Glow-type luminescence was recorded after 10 min using a FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg Germany) multimode reader. The activity is proportional to the difference in the total and consumed ATP. The inhibitory activities were calculated on the basis of maximal activities measured in the absence of inhibitor. The IC<sub>50</sub> was defined as the concentration of each compound that reduces a 50 % the enzymatic activity with respect to that without inhibitors. Five different concentrations by duplicate were used to determine the dose/response curve.

**Cell viability.** SH-SY5Y cells were seeded in a 96-well plate at  $2 \times 10^4$  cells/well, incubated for 24 h and afterwards treated with different concentrations of curcumin-DEF hybrids 4-7, TDZD and DMF (1.25–40  $\mu$ M) for 24 h at 37 $^{\circ}$ C in 5 % CO<sub>2</sub>. Cell viability, in terms of mitochondrial metabolic function, was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, as previously described (see Supporting Information for experimental details).<sup>2</sup>

**Detection of phosphorylated GSK-3 $\beta$  kinase by Western blotting.** SH-SY5Y cells were seeded in 60 mm dishes at  $2 \times 10^6$  cells/dish, incubated for 24 h and subsequently treated with curcumin-DEF hybrids **5** and **6** (5  $\mu$ M) for 1, 3 and 6 h at 37 $^{\circ}$ C in 5 % CO<sub>2</sub>. At the end of incubation, cells were lysed by the addition of ice-cold lysis buffer containing leupeptin 2  $\mu$ g/mL and PMSF 100  $\mu$ g/mL. An aliquot was used for protein analysis with the Bradford assay for protein quantification. Cell lysates (50  $\mu$ g per sample) were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes, which were probed with primary phospho-GSK3 $\alpha/\beta$  (Ser21/9) (1:1000; Cell Signaling Technology, Danvers, MA, USA) and secondary antibodies. ECL reagents (Pierce, Rockford, IL, USA) were utilized to detect targeted bands. The same

membranes were stripped and reprobed with GSK-3 $\beta$  (1:1000; Cell Signaling Technology) and  $\beta$ -Actin (1:1000; Sigma Aldrich) antibodies. Data were analysed by densitometry, using Quantity One software (Bio-Rad, Hercules, CA, USA). Data are expressed as a ratio between phospho-GSK3 $\alpha/\beta$  and GSK3 $\beta$  levels normalized against  $\beta$ -Actin. Data were analysed by densitometry, using Quantity One software (Bio-Rad). Data are expressed as fold increase versus respective contralateral intact site.

**BBB permeation.** Prediction of the brain penetration was evaluated using a parallel artificial membrane permeability assay (PAMPA).<sup>1</sup> Ten commercial drugs, phosphate buffer saline solution at pH 7.4 (PBS), Ethanol and dodecane were purchased from Sigma, Acros organics, Merck, Aldrich and Fluka. The porcine polar brain lipid (PBL) (catalog no. 141101) was from Avanti Polar Lipids. The donor plate was a 96-well filtrate plate (Multiscreen<sup>®</sup> IP Sterile Plate PDVF membrane, pore size is 0.45  $\mu$ M, catalog no. MAIPS4510) and the acceptor plate was an indented 96-well plate (Multiscreen<sup>®</sup>, catalog no. MAMCS9610) both from Millipore. Filter PDVF membrane units (diameter 30 mm, pore size 0.45  $\mu$ m) from Symta were used to filtered the samples. A 96-well plate UV reader (Thermoscientific, Multiskan spectrum) was used for the UV measurements. Test compounds [(3-5 mg of Caffeine, Enoxacin, Hydrocortisone, Desipramine, Ofloxacin, Piroxicam, Testosterone), (12 mg of Promazine) and 25 mg of Verapamil and Atenolol] were dissolved in EtOH (1000  $\mu$ L). 100 microlitres of this compound stock solution was taken and 1400  $\mu$ L of EtOH and 3500  $\mu$ L of PBS pH =7.4 buffer were added to reach 30% of EtOH concentration in the experiment. These solutions were filtered. The acceptor 96-well microplate was filled with 180  $\mu$ L of PBS/EtOH (70/30). The donor 96-well plate was coated with 4  $\mu$ L of porcine brain lipid in dodecane (20 mg mL<sup>-1</sup>) and after 5 minutes, 180  $\mu$ L of each compound solution was added. 1-2 mg of every compound to be determined their ability to pass the brain barrier were dissolved in 1500  $\mu$ L of EtOH and 3500  $\mu$ L of PBS pH=7.4 buffer, filtered and then added to the donor 96-well plate. Then the donor plate was carefully put on the acceptor plate to form a “sandwich”, which was left undisturbed for 2h and 30 min at 25 °C. During this time the compounds diffused from the donor plate through the brain lipid membrane into the acceptor plate. After incubation, the donor plate was removed. UV plate reader determined the concentration of compounds and commercial drugs in the acceptor and the donor wells. Every sample was analyzed at three to five wavelengths, in 3 wells and in two independent runs. Results are given as the mean [standard deviation (SD)] and the average of the two runs is reported. 10 quality control compounds (previously mentioned) of known BBB permeability were included in each experiment to validate the analysis set.

## References

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2. Rampa, A.; Tarozzi, A.; Mancini, F.; Pruccoli, L.; Di Martino, R. M.; Gobbi, S.; Bisi, A.; De Simone, A.; Palomba, F.; Zaccheroni, N.; Belluti, F. (2016) Naturally Inspired Molecules as Multifunctional Agents for Alzheimer's Disease Treatment. *Molecules.* **21**, 643.