Supplementary Information for

Amyloid binding and beyond: a new approach for Alzheimer's disease drug discovery targeting Aβo - PrP[C] binding and downstream pathways

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Methods

The experimental section is composed of three interconnected domains: computational design, NMR-based biophysical screening and cellular models for biochemical and functional evaluations. The relationship amongst these domains is shown in a flow chart (**Supplementary Figure 1**).

Ethical statement: Studies using human induced pluripotent stem cells were performed in strict accordance with the European Framework 7 Marie Curies guidelines. They are either commercially certified or approved by local institutions.

Computational Design

Designing fluorinated compound libraries

Creation of seed compounds from known active anti-Aβ binders in literature and in database

Published inhibitors of Aβo aggregation or binders were extracted from the dataset deposited by Joshi et al[1] and used as seed compounds. This yielded 89 compounds. Additional seed compounds were obtained from the ChEMBL collection by searching the ChEMBL target ID 2487 which is the Aβ protein. Resulting compounds were discarded from the collection[2, 3] if they did not have an activity measurement or if the operator was missing or uncertain (e.g. >, <, ~) leaving 62 compounds with measured K_i, K_d, or IC_{50} values. Compounds from literature and the database search were combined and formed a collection of 151 seed compounds. **(Supplementary Table 1)**

Fragmentation of the seed compounds

The Dicer algorithm (https://github.com/EliLillyCo/LillyMol) was then used to fragment the seed compounds to generate all fragments of greater than 6 heavy atoms from cutting one or two acylic single bonds between heavy atoms in each of the input set of compounds. An example is shown in Figure 1 in the main paper. In total, 813 fragments were generated and these fragments were used in the subsequent substructure matching search.

Database construction

A series of non-proprietary compounds available within Lilly's internal compound stores was identified by cross-referencing ChEMBL 21[2, 4], DrugBank 5.0.1, the "All clean" subset of ZINC 12[5] and PubChem[6] (accessed September 8th, 2016) with the structures of all compounds with current Lilly inventory. This yielded a database of 73, 848 compounds. A sub-set of 7,220 library compounds was selected by requiring that library compounds have at least one fluorine atom which was used for the ^{19}F NMR screen.

Generation of fluorine-containing small molecules as potential Aβo binders by screening of fragments against fluorine sub-set of the database

The 813 fragments generated by fragmentation of the seed compounds were used to perform a substructure matching search against the database of 7,220 compounds using RDKit. From the set of 4,142 initial hits, 2000 diverse compounds were selected. These compounds were further filtered by predicted solubility. Compounds with predicted solubilities smaller than 0.1 mg/mL were excluded which yielded a diverse subset of 971 compounds for experimental screening by the ^{19}F NMR **(Supplementary Table 2)**.

Hit expansion

A similarity search was performed on the 8 initial hits confirmed by ^{19}F NMR screening in the follow-up hit expansion exercise in order to identify more active compounds. The similarity search was performed against the whole database of 73,848 compounds. The top five most similar compounds by Tanimoto similarity score (using the GFP fingerprints) for each of the 8 hit compounds were retained which gave 33 analogues for NMR validation after duplicates were removed.

Biophysical validation of virtual Aβo binders using NMR techniques

Preparation of Aβ oligomers(Aβo) from synthetic Aβ$_{1-42}$ monomers for NMR experiment

Aβ oligomers were prepared as described previously with some modifications.[7, 8] Briefly, 16 mg of lyophilized human Aβ$_{1-42}$ peptide was equilibrated to room temperature for 30 minutes prior to resuspension in hexafluoro-2-propanol (HFIP) to produce a 4.5 mg/ml Aβ solution. The peptide solution was incubated at room temperature for a further 30 minutes to ensure complete dissolution. The resulting peptide solution was then aliquoted into 0.5 mg aliquots in LoBind microcentrifuge tubes, centrifuged at maximum speed for 5 minutes, and dried under N$_2$ to remove HFIP for 1 hour. The dried peptide films were stored at -20 ºC in a desiccator jar. Aβ$_{1-42}$ peptide films were removed from the freezer 10 minutes prior to use to equilibrate to room temperature. HiTrap desalting columns (5 mL; GE Healthcare) were carefully equilibrated with 20 mL of Neurobasal Medium (Gibco by Life Technologies). Each peptide film was resuspended in 250 μL of DMSO to yield a final Aβ concentration of 10 mM and were vortexed and pooled prior to adding to the column. DMSO was removed from the peptide by passing the solution through the HiTrap column, and the peptide was eluted into two volumes of 1 mL of neurobasal medium passed through the column. The eluted peptide was collected into LoBind tubes and protein concentration was assessed via a Bradford protein assay kit (Thermo Fisher Scientific). Each tube was normalized with neurobasal medium to a final concentration of 220 μg/ml (50 μM) and split equally into LoBind tubes to allow an equal ratio of liquid:air (e.g. 1 ml peptide in a 2 ml tube). The normalized Aβ$_{1-42}$ monomers were then oligomerized for 1 hour at 25 ºC using a plate shaker at 330 rpm. Finally, the oligomers were centrifuged for 10 minutes at 14,500 rpm, the supernatant was collected and analysed using a DynaPro Dynamic Light Scattering (DLS) Instrument (Wyatt Technology), and the results analysed using Dynamics V7 Software. The prepared Aβo were stored at -80 ºC and defrosted prior to NMR experiments.

Characterization of Aβo using DynaPro Dynamic Light Scattering (DLS)

The freshly prepared Aβo was analysed using DynaPro Dynamic Light Scattering (DLS) Instrument (Wyatt Technology). Freshly prepared Aβo (200 μL, 0.05 mM solution in neurobasal media) was added to a clear bottom 96 well plate ensuring no bubbles had formed. The DLS instrument was pre-set at a constant temperature of 25 °C and solvent set to neurobasal media. Standard settings of 5 second acquisition and 5-minute wait time were used for each scan. The data was analysed within the DYNAMICS software. Aβo produced with less than 50% mass of radii between 10-20 nm were discarded.

Preparation of the scrambled Aβ polypeptide for NMR experiment

A scrambled Aβ polypeptide of the same amino acid composition as Aβ$_{1-42}$, but different primary sequence from that of Aβ$_{1-42}$, AIAEGSHVLKEGAYMEIFDVQGHVFGGKIFRVVDLGSHNVA, was purchased as a synthetic peptide from CPC scientific. The peptide was subjected to the same procedure as detailed for Aβ described above. The prepared peptide was not subjected to DLS characterisation as it is non-amyloidal peptide.

NMR experiments

Ligand stock solution

All ligands tested were sourced from Lilly's internal inventory as a 10 mM solution in DMSO and used without further purification. Reference compounds, Bexarotene is sourced from Lilly's internal inventory; flurbiprofen and ibuprofen are purchased from Sigma-Aldrich.

Preparation of deuterated phosphate-buffered saline solution

Standard phosphate-buffered saline (PBS Buffer) solution (10 mL) was frozen until solid in a dry ice acetone bath before being transferred to a -80 freezer for a minimum of 6h. The frozen solution was put under vacuum overnight until a fine white powder remained. D$_2$O (10 mL) was then added immediately to reconstitute the buffer solution. The deuterated PBS buffer was used for all STD-NMR experiments.

General procedure

All NMR experiments were performed on a Bruker Avance III 500 MHz or 600 MHz spectrometer equipped with a prodigy or QCI cryoprobe respectively.

^{1}H NMR experiments were carried out using a standard Bruker protocol.

^{19}F CPMG experiments were performed using a standard pulse sequence with ^{1}H decoupling during acquisition and a 400 ms CPMG time. Typically, 128 transients were acquired. Spectra were processed with 1 Hz exponential line broadening and peak areas extracted using a custom python script for topspin.

For STD experiments, a standard Bruker pulse sequence was used, with 2 s of saturation at 0ppm and -40 ppm for on and off resonance. Double solvent suppression was achieved with a DPFGSE element incorporating a selective 180 pulse designed to flip both the water (from buffer) and dmso (from ligand stock) peaks. Typically, 128 scans were acquired prior to processing with a 1 Hz line broadening function. Data was processed in topspin using the stdsplit AU program.

^{1}H-NMR for all 971 compounds from virtual design

The corresponding ligand (6 µL, 10 mM solution in DMSO) was diluted with standard PBS buffer (1200 µL) providing a ligand concentration of 0.05 mM. The ligand (550 µL, 0.0485 mM solution) was added to one 5mm NMR tube.

^{1}H-NMR validation of binding between Aβo and bexarotene

Bexarotene (6 µL, 10 mM solution in DMSO) was diluted with standard PBS buffer (1200 µL) providing a ligand concentration of 0.05 mM. Bexarotene (550 µL, 0.0485 mM solution) was added to two 5mm NMR tubes. Aβo (11 µL, 0.05 mM solution in neurobasal media) was added to one tube providing an active Aβ concentration of 0.001 mM. Neurobasal media (11 µL) was added to the second tube providing a control.

¹⁹F NMR validation of binding between BSA and flurbiprofen

Flurbiprofen (0.100 g, 0.401 mmol) was diluted with DMSO (40.1 µL) to produce a 10 mM solution. Flurbiprofen (6 µL, 10 mM solution in DMSO) was diluted with standard PBS buffer (1200 µL) providing a ligand concentration of 0.05 mM. The flurbiprofen solution (550 µL, 0.0485 mM solution) was added to two 5mm NMR tubes. BSA (11 µL, 0.05 mM solution in neurobasal media) was added to one tube providing a BSA concentration of 0.001 mM. Neurobasal media (11 µL) was added to the second tube providing a control.

Primary ¹⁹F NMR screening of fluorine containing virtual hits from computational design
Screening of sub-library containing 6 compounds
Each ligand (6 µL, 10 mM solution in DMSO) was contained within a mixture was diluted with standard PBS buffer (1200 µL) providing an individual ligand concentration of 0.0485 mM. The ligand (550 µL, 0.0485 mM solution) was added to a 5mm NMR tube. Aβo (13 µL, 0.05 mM solution in neurobasal media) was added providing an Aβo concentration of 0.001 mM.

Screening of sub-library containing 7 compounds
Each ligand (6 µL, 10 mM solution in DMSO) contained within a mixture was diluted with standard PBS buffer (1200 µL) providing an individual ligand concentration of 0.0483 mM. The ligand (550 µL, 0.0485 mM solution) was added to a 5mm NMR tube. The Aβo (13 µL, 0.05 mM solution in neurobasal media) was added providing an Aβo concentration of 0.001 mM.

¹⁹F NMR procedure for validation of binding of individual hits from initial sub-library screening (Aβo)
The corresponding hit compound (6 µL, 10 mM solution in DMSO) was diluted with standard PBS buffer (1200 µL) providing a compound concentration of 0.05 mM. The ligand (550 µL, 0.0485 mM solution) was added to two 5mm NMR tubes. The Aβo (11 µL, 0.05 mM solution in neurobasal media) was added to one tube providing an Aβo concentration of 0.001 mM. Neurobasal media (11 µL) was added to the second tube providing a control.

¹⁹F NMR procedure for validation of specific binding of initial hits using scrambled Aβ peptide
The corresponding hit compound (6 µL, 10 mM solution in DMSO) was diluted with standard PBS buffer (1200 µL) providing a ligand concentration of 0.05 mM. The ligand (550 µL, 0.0485 mM solution) was added to two 5mm NMR tubes. The Aβo (11 µL, 0.05 mM solution in neurobasal media) was added to one tube providing an Aβo concentration of 0.001 mM. Scrambled Aβ peptide (11 µL, 0.05 mM solution in neurobasal media) was added to the other tube providing a scrambled Aβ peptide concentration of 0.001 mM.

¹⁹F NMR hit expansion competition assay
The corresponding 'seed' compound (6 µL, 10 mM solution in DMSO) was diluted with standard PBS buffer (1200 µL) providing a 'seed' ligand concentration of 0.05 mM. Active Aβ (24 µL, 0.05 mM solution in neurobasal media) was added to the 'seed' ligand solution providing an active Aβ concentration of 0.001 mM. The 'seed' ligand/ Aβo solution (600 µL, 0.0485 mM ligand, 0.001 mM active Aβ) was added to two 5mm NMR tubes. The

corresponding competing ligand (3 µL, 10 mM solution in DMSO) was added to one NMR tube providing a concentration of 0.05 mM. DMSO (3 µL) was added to the second tube providing a control.

STD NMR Validation

STD NMR validation between BSA and Ibuprofen

BSA (1.00g, 0.014 mmol) was diluted with neurobasal media (289 µL) to produce a 0.05 mM solution. BSA (200 µL, 0.05 mM solution in neurobasal media) was diluted with a deuterated PBS buffer (4800 µL) providing a BSA concentration of 0.002 mM. BSA (550 µL, 0.002 mM) was added to a 5mm NMR tube. Ibuprofen (0.100 g, 0.485 mmol) was diluted with DMSO (48.5 µL) to produce a 10 mM solution. Ibuprofen (11 µL, 10 mM solution in DMSO) was added providing a concentration of 0.2 mM.

STD-NMR validation of the binding of selected hits and Aβo

The Aβo (200 µL, 0.05 mM solution in neurobasal media) was diluted with a deuterated PBS buffer (5000 µL) providing an Aβo concentration of 0.002 mM. The Aβo (550 µL, 0.002 mM) was added to a 5mm NMR tube. The corresponding compound (11 µL, 10 mM solution in DMSO) was added providing a concentration of 0.2 mM.

STD-NMR procedure for the control compounds

Neurobasal media (200 µL) was diluted with a deuterated PBS buffer (4800 µL) and the control solution (550 µL) added to a 5mm NMR tube. The corresponding ligand (11 µL, 10 mM solution in DMSO) was added providing a concentration of 0.2 mM.

STD-NMR dose response studies

Active Aβ (4 µL, 8 µL, 16 µL, 24 µL, 32 µL, 40 µL, 48 µL, 0.05 mM solution in neurobasal media) was diluted with deuterated PBS buffer (196 µL, 192 µL, 184 µL, 176 µL, 168 µL, 160 µL, 152 µL) providing an active Aβ concentration of 0.001 mM, 0.002 mM, 0.004 mM, 0.006 mM, 0.008 mM, 0.01 mM, 0.012 mM respectively. The active Aβ (200 µL, 0.001 mM, 0.002 mM, 0.004 mM, 0.006 mM, 0.008 mM, 0.01 mM, 0.012 mM) concentrations were added to seven 3mm NMR tubes. The corresponding ligand (4 µL, 10 mM solution in DMSO) was added to all seven NMR tubes providing a ligand concentration of 0.2 mM.

Biological evaluation of hit compounds

Preparation of recombinant Aβo from CHO cell line for cellular functional assay

Expression of the recombinant Aβo using 7PA2 CHO cell line

A solution containing recombinant amyloid-beta oligomers (rAβo)was obtained from the conditioned medium of 7PA2 cells, a Chinese Hamster Ovary cells stably transfected with cDNA encoding APP751, an amyloid precursor protein that contains the Val717Phe familial Alzheimer's disease mutation.[9] In order to obtain rAβo, $5x10^6$ transfected 7PA2 cells were seeded in a T175 flask and cultured in Dulbecco's modified Eagle's medium (DMEM, sigma) supplemented with 10% foetal bovine serum (ThermoFisher), 2 mM L-glutamine (Sigma-Aldrich) 50 mg/ml penicillin/streptomycin. The cells were incubated for 24 hours in 5% CO2 at 37°C. After 24 h of incubation the cells were washed with serum-free medium and conditioned in 5 mL of plain DMEM without phenol red (ThermoFisher) and lacking any additives, overnight. The oligomer-containing conditioned medium (CM) was collected and cleared of cells and debris by centrifugation at 200 g for 10 min at 4°C. The CM was used as the natural Aβo solution in the fear conditioning experiments for HCS. The concentrated CM contained between 1,000– 2,000 pg/ml of Aβo as measured by ELISA (ThermoFisher).

Quantification of Aβo by ELISA

The levels of soluble Aβo, in the supernatant of the above conditioned medium (CM) obtained from the harvest of the transfected 7PA2 cells, was measured by enzyme-linked immunosorbent assay (ELISA) kit (KHB3441 for $A\beta_{1-42}$; Invitrogen, Carlsbad, CA, USA). Briefly, the protein standards of known concentration of $A\beta_{1-42}$ provided in the kit were mixed with detection antibody and loaded onto the antibody-pre-coated plate at the designated wells. After incubation, the samples plate? was washed and horseradish peroxidase-conjugated secondary antibody was added to all plates, and the substrates were added for colorimetric reaction, which was stopped with sulfuric acid. Optical density was obtained, and a standard curve was constructed. Samples from the supernatant were treated in the same way as the protein standards and the optical densities were measured. The concentrations of Aβo were extrapolated from the standard curve. The Aβo concentration measured is in the range of 1,000– 2,000 pg/ml by ELISA.

Preparation of the stock solution

Six compounds (**CHEMBL1673279, PBCHM4680099, PBCHM57487213, ZINC00120199, PBCHM120765, and PBCHM81560982**) were selected for the biological evaluation using cellular assays. The purity of the compounds was greater than 95% assessed by LC-MS (LC protocol: column - Gemini NX C18 3μ 2 x 50 mm; flow rate: 1.2mL/min at 50˚C; Solvent A: water + 0.1% formic acid B: CH_3CN + 0.1% formic acid; Gradient 5>95%B in 1.5min + 0.5min hold; MS protocol: Agilent 1290 equipped with a single quad mass spectrometer. The MS scan range was 100-800 m/z) except 90% in purity for compound **ZINC00120199**. 10 mM DMSO (Sigma Chemical Co) stock of each compound was prepared for use in the subsequent cellular assays and kept out of light at -20°C until further use. The control compound, PP1 (Potent, selective Src family kinase inhibitor) was obtained from Tocris (1397) stored at 1 mM in dimethyl sulfoxide (DMSO; Sigma Chemical Co), and kept out of light at -20°C until use.

Validation of the suitability of HEK297 as a cellular Aβo-PrP^C binding model

Neural gene expression level in selected human cell lines

HEK cell, icells and SH-SY5Y cell samples were collected during routine subculture. Three separate samples were taken, each containing approximately 1 x 10^6 cells. The samples were spun down, the medium removed and the pellet washed with 100 μl PBS. Cell pellets of each cell type were resuspended in 350 μl RLT Buffer and was aspirated to resuspend, and the suspension transferred to a gDNA spin column which was placed in a 2 ml collection tube. The tube was spun for 30 secs at >8,000 g. The column was discarded and the flow-through (FT) solution was collected. 350 μl of ethanol was added to the FT solution and mixed well by pipetting. The solution was transferred to an RNeasy spin column in a 2 ml collection tube and spun for 15 secs >8,000g and FT solution was discarded. 700 μl RW1 buffer (from kit) was added and the column was spun for 15 secs at >8,000 g and FT discarded. 500 μl RPE was added and the column was spun for 15 secs at >8,000g, and FT discarded, and the process was repeated 3 times. The RNeasy column was then placed in a fresh 2 ml collection tube and spun for 1 minute at 8,000g to remove any remaining organic impurities. The column was eluted using 50 μl RNAse-free water and eluent was collected by centrifugation for 1 min >8,000 g. For every 100 μl of eluent collected, 11 μl reaction buffer and 1 μl DNase were added, incubated for 30 mins at 37 ℃. 5 μl DNase inactivation reagent (beads) was then added to the solution and incubated for 5 mins with occasional mixing. The supernatant was transferred (~95 μl for 100 μl eluent) to a clean tube after centrifugation to remove the beads and debris. 2 μl of solution from each cell type was used to measure the concentration of the RNA extracted from each cell type using nanodrop.

The prepared RNA was used to generate cDNA required for the qPCR experiment. Briefly, two qPCR plates, one for PRNP, FYN, GRIN1, GRIN2A, GRIN2B, MAPT, GAPDH and PSMB2 and the other for BACE1, GRM5, GAPDH and PSMB1, were used in RT reaction. The master mix for RT reaction is composed of 1 mM dNTPs, 2.5 μM hexamers, 0.4U/μl RNase inhibitor, 2U/μl MMLV-RT in buffer. 23.2 μL of RT mix, volume that equivalent to 400 ng of RNA and nuclease free water was added to each well to give a final reaction volume of 80 μL. All wells were mixed with a multi-channel pipette, checked to ensure there are no air bubbles and tightly sealed. The G-storm cycler was used to run the RT reaction. The cDNA prepared is ready for qPCR analysis.

The cDNA samples were split equally into 4 portions and placed in a 384-well plate so that each sample is run in quadruplicate. The qiagen robot mixes the cDNA with the primers and the taqman master mix and brings the reaction up to the correct volume and concentration with nuclease free water to a total reaction volume of 80 μL. The plate was securely sealed and then briefly centrifuged to get rid of any air bubbles. Standard Applied Biosystem Wizard was used for the qPCR experiment.

The subsequent data analysis was done using PSMB2 only as the reference gene. The data is presented as a Ct value which is the number of cycles a particular sample has been subjected to at the point where fluorescence levels cross the threshold. As 2 plates were being compared (for the human samples) the threshold was set manually at 0.2. The Ct values were converted to ΔCt by subtracting the Ct for the target gene from the Ct for the housekeeping gene from the same sample. The ΔCt value was then converted to a ΔΔCt value by subtracting it from the calibrator gene, in this case PRNP in HEKs (for human samples) or glia (in rat samples). The ΔΔCt was then converted into a percentage of the calibrator protein by calculating the 2^-ΔΔCt.

Analysis of PrP^C expression level by flow cytometry

The cultured cells were then resuspended in an ice cold flow cytometry buffer (PBS Ca^{++}/Mg^{++}, 10% FBS, 1% sodium azide) 1 mL for 5-10 mins. Sodium azide prevents the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity. The primary anti-prion 8H4 (conc. 0.01 M) was diluted 1:200 in 100 µl PBS 5% BSA and then the cells were resuspended in this solution and incubated for 1 hours on ice and protected from light. The same was done for the Isotype (conc. 2.5 mg/mL) 0.8:200. The cells were washed two times with cold PBS Ca^{++}/Mg^{++} by centrifugation at 2000 rpm for 5 min and incubated with the secondary antibody in dark for 1 hour under gentle agitation. The fluorochrome-labeled secondary antibody Alexa Fluor 488 was diluted 1:500 in 100 µl PBS 5% BSA. Before the analysis the cells were washed and resuspend in 300 µL cold PBS Ca++/Mg++ and 2 µL of Propidium Iodide (PI) added to each sample to exclude dead cells. For best results, all reagents/solutions used were kept cold and cells kept on ice and analyzed immediately on the flow cytometer

Validation of Aβo binding to PrP^C on HEK293 cells by anti-PrP antibody using ICC

4 x 10^4 HEK 239 cells were seeded and incubated overnight. The medium was removed and replaced with DMEM phenol red free, because it is known that phenol red binds Aβ creating a high background during the analysis. HEK 293 cells were treated with recombinant Aβo1000 pg/mL for 2 hours. After the incubation with treatment, the media was aspirated, and cells washed by 1 mL PBS containing Ca^{++}/Mg^{++}. 500 µL of 4% PFA were added in each well, to fix the cells, and incubate 15 min at RT. PFA was removed and cells washed with 500 µL PBS 1 time. If the cells had to be stored this was done twice and cells stored in the fridge with PBS. The cells were blocked with PBS-T 5% donkey serum for 1h at RT and the coverslips transferred on a candle wax support. The blocking solution was aspirated, and the control was incubated with primary antibody anti-prion 6D11 and the samples with anti-amyloid β antibody 6E10 overnight at 4 °C. How about DAPI and Phallidine staining? The antibodies were made up in 50 µL PBS-T 1%BSA with a dilution factor of 1:250. The primary antibodies were washed away 3 times with 50 µL PBS-T 5 min each at RT and the secondary antibody anti-mouse Alexa fluor 488 or 594 was added to each well and incubate for 1h at RT. The antibodies AF488 (green) and AF594 (red) were made up in PBS-T 5% donkey serum with a dilution factor of 1:400 and 1:500 respectively. After one hour the secondary antibodies were aspirated and coverslips washed 2 times with PBS-T and 1 time with PBS. 50 µL of DAPI 100 ng/mL was added to each coverslip and incubated for 5 min. Cover slips were washed 3 times with PBS before to be mounted on the slide, previously treated with 5 µL of propyl galate on the coverslip position and sealed by using clear nail varnish to prevent contamination of the microscope with biohazardous material. The slides can be analysed immediately or stored in the fridge for days. With the confocal microscopes the nuclei stained with DAPI were visualized under excitation with Diode 50 mW with 405 nm emission line (UV). The PrP^C and Aβ oligomers, bounded to it, stained with AF488 and AF594 were visualized after excitation with "Blue" argon multi-line 65 mW with 488 nm emission line (visible) and "Yellow" diode 20 mW with 561 nm emission line (visible) respectively. Images were analyzed using ImageJ open source software.

Effects of hit compounds upon the disruption of the binding between Aβo and PrP^C using HEK293 cell line

MTT cytotoxicity assay.

HEK cells were seeded onto a 96-well plate at a density of 1 x 10^4 cells per well and incubated at 37 °C with 5% CO_2 overnight prior to the assay. Each compound was added in DMSO to reach final concentrations of 1 μM, 5 μM, 10 μM, 20 μM and 50 μM, in triplicates, adjusting the final DMSO concentration to 0.5%. After 24 hours of incubation at 37 °C, 5% CO_2, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 mg/ml in PBS) were added to each well, and cells were incubated for another 2 hours. Then, the medium was removed, and the blue formazan crystals dissolved in 60 μL of acidified isopropanol. The plates were shaken to promote full dissolution of the crystals, after which the optical density (OD) at 570 nm (with a 690 nm reference filter) was measured using a microplate reader. Cell survival rates were calculated using equation (**1**), and the results presented as the average of two experiments performed in triplicates.

$$Cell\ Survival\ (\%) = \left[\frac{OD_{sample} - OD_{medium}}{OD_{cell\ control} - OD_{medium}}\right] x\ 100 \quad (1)$$

Effects of hit compounds upon the disruption between Aβo-PrP^C binding using Immunocytochemistry
96-well plates were incubated with 50 μL of poly-L-ornithine hydrobromide 100 μg/mL for 40 minutes and washed with PBS 3 times. HEK cells were seeded onto the plates diluted in phenol red-free DMEM medium with high glucose (Gibco) to achieve 2 x 10^4 cells per 100 μL in each well. The cells were incubated at 37 °C with 5% CO_2 overnight. Then, the medium was removed and 50 μL of conditioning medium with 1000 pc/mL of above prepared recombinant Aβo was added to each well. After a 2 hour-long incubation period, the conditioning medium was removed and the cells were washed once with PBS. Then, 100 μL of fresh phenol red-free DMEM medium with high glucose were added to each well and the cells were incubated with the hit compound at the desired concentration (10 μM in the screening assay or 1 μM – 20 μM in the dose-response experiment) in 0.5% of DMSO for 1 hour. The medium was subsequently removed. The cells were washed with 100 μL of PBS containing Mg^{2+} and Ca^{2+}, after which 100 μL of 4% PFA were added to each well. Once incubated for 15 minutes at room temperature, the PFA was removed and the cells were washed once again with 100 μL of PBS containing Mg^{2+} and Ca^{2+}. 100 μL of PBS-T with 5% of donkey serum were added and the cells were incubated for 1 hour at room temperature. Then, the blocking solution was removed and the primary antibody (anti-Aβo 6E10 antibody prepared in 50 μL of PBS-T with 5% of donkey serum, 1:250 dilution) was added prior to overnight incubation at 4 °C. After removal of the primary antibody, the cells were washed 3 times with 50 μL of PBS-T for 5 minutes at room temperature. 50 μL of the secondary antibody (Alexa fluor 594 anti-mouse antibody, prepared in PBS-T, 1:500 dilution) were subsequently added to each well and the cells were incubated another hour at room temperature. The liquid was aspirated, and the cells were washed twice with PBS-T and once with PBS (50 μL each wash). 50 μL of 4′,6-diamidino-2-phenylindole (DAPI, 100 ng/mL in PBS) were added to each well. Following 5 minutes of incubation, DAPI was removed and the cells washed with 100 μL of PBS 3 times. 100 μL of PBS were finally added and the imaging was carried out by The Wolfson Light Microscopy Facility, using ImageXpress Micro Widefield High Content Screening System, 20X magnification, and 30 pictures taken per each well. Data analysis was executed using MetaXpress Software Multi-Wavelength Translocation Application Module. Results are presented as the average of two experiments performed in triplicates.

Effects of hit compounds upon pFyn and pTau using human iPSC derived neural progenitor cells (NPC) and mature cortical neurons hiPSC culturing

The human induced pluripotent stem cells (hiPSCs) line derived from a health individual used in this study iPS (control MIFF1, https://web.expasy.org/cellosaurus/CVCL_1E69)[10] was kindly provided by Professor Peter Andrews and Dr. Ivana Barbaric (Centre for Stem Cell Biology, The University of Sheffield). hiPSCs were grown and maintained on Vitronectin-coated plates ($0.5\mu g/cm^2$; ThermoFisher Scientific) according to the manufacturer's recommendations in complete TeSR™-E8™ Medium (StemCell Technologies). Culture medium was changed every day. Cells were passaged every 5-7 days as clumps using ReLeSR™ an enzyme-free reagent for cell dissociation (StemCell Technologies) according to the manufacturer's recommendations. For all the experiments in this study, hiPSCs were used between passage 18 and 26, all iPSCs were cultured in 5% O_2, 5% CO_2 at 37°C.

Neural differentiation

Neural induction of hiPSCs (Supplementary Figure 2) was performed using the modified version of dual SMAD inhibition protocol. hiPSCs were detached by 3 minutes of incubation with Versene Solution (Gibco®), after incubation the solution was removed and 1mL of complete TeSR™-E8™ Medium (StemCell Technologies) was added per well of 6 well plate and detached with a cell lifter (Corning) and then the cell suspension was transferred for Matrigel-coated plate (Corning® Matrigel® Growth Factor Reduced). On the day after plating (day 1), after the cells have reached ~100% confluence, the cells were washed once with PBS and then the medium was replaced for neural medium (50% of DMEM/F-12, 50 % of Neurobasal, 0.5× N2 supplement, 1x Gibco® GlutaMAX™ Supplement, 0.5x B-27, 50 U ml−1 penicillin and 50 mg ml−1 streptomycin, supplemented with SMAD inhibitors (SMAD-i, DMH-1 2 μM SB431542-10 μM TOCRIS). The medium was changed every day for 7 days. On day 8, when a uniform neuroepithelial sheet was observed, the cells were split 1:1 with Accutase (StemPro® Accutase® Cell Dissociation Reagent, Gibco™ A1110501), onto matrigel substrate in the presence of 10 μM of Rock inhibitor (Rock-i, Y-27632 dihydrochloride, Tocris), giving rise to a sheet of neural progenitor cells (NPC). After 24 hours of incubation the medium was removed and replaced for neural medium without Rock-i. Culture medium was changed every second day and cells were split once they reached 100% of confluence.

Neuronal induction from neural progenitor cells (NPC) was obtained by modified from previously described methods.[95] NPCs were transferred to poly-L-ornithine/laminin-coated plates (10 μg /mL) and the medium was replaced for neuronal medium (Neurobasal medium, 1x Gibco® GlutaMAX™ Supplement, 1x B-27), supplemented with DAPT 10 μM. The medium was changed every day for 6 days, immature neurons emerged around day 26. The cells were continuous cultured in neuronal medium as above described until day 40 when the young neurons were split with accutase onto to poly-L-ornithine/laminin-coated plates (10 μg /mL) and the medium was replaced for neuronal medium without DAPT and supplemented with 10nM of BDNF. The cells were then fed on alternate days with neuronal medium until day 75. The characterization of NPC and neurons can be seen below.

Electrophysiological characterization of hiPSC derived cortical neurons by patch clamp technique

Patch clamp electrophysiology was used to study the firing properties and help to confirm the maturation of hiPSC-derived cortical neurons. Culture slides were collected from 24-well culture plates. Whole-cell patch-clamp recordings were performed at 8–10 weeks following the initiation of NPC differentiation. Neurons with a large cell body and neurite-like structures were chosen for examination. Recording micropipettes (tip resistance

3–6 MΩ) were filled with internal solutions. Recordings were made at room temperature using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). We injected current every 2pA through a current clamp (CC) protocol on these neurons. One control (n=16 cells) was used for the experiments. The whole-cell capacitance was compensated, and series resistance was monitored throughout the experiment in order to confirm the integrity of the patch seal and the stability of the recording. Voltage was corrected for liquid junction potential. For voltage-clamp recordings, cells were clamped at − 70 mV. Spontaneous postsynaptic currents were recorded for 3 min. Fast sodium and potassium currents were evoked by voltage steps ranging from − 70 to +50 mV in 10 mV increments. Capacitance was derived from the Clampex 10.2 (Molecular Devices) membrane-test function. For current-clamp recordings, voltage responses were evoked from a holding potential of − 75 mV using 500 ms steps ranging from − 20 to +150 pA in 10 pA intervals delivered at 0.5 Hz. Single action potential (AP) properties were calculated from the first evoked AP in response to a depolarizing step.

Compound treatments

The cells were exposed to the solution containing natural Aβ oligomers obtained from 7PA2 cells (1,000 pg/ml of Aβo). To determine the effects of the compounds on inhibit activation of FYN Kinase, NPC-cultures were pre-treated for 45 minutes with 10 µM of the compounds or 1 µM of PP diluted in neurobasal medium without phenol red. After pre-treatment the cells were exposed to 1,000 pg/mL of Aβ oligomers in association with the compounds for 15 minutes; control cultures were treated with DMSO the vehicle of dilution of the drugs. PP1 was used as a control of inhibition of FYN activation. In order to evaluate the potential of the compounds in inhibiting the hyper phosphorylation of tau cortical neurons were exposed to 1,000 pg/ml of Aβo in association with 10 µM of the compounds for 5 days.

Immunofluorescence

For immunostaining, hiPSCs, NPC and neurons were washed with phosphate- buffered saline (PBS) and fixed by immersion in 4% paraformaldehyde for 15 minutes at room temperature. Following fixation, samples were washed three times with PBS and permeabilized with 0.3% Triton X-100 in PBS (Sigma) for 5 min in order to detect intracellular antigens. After permeabilization, cells were blocked by incubation with PBS containing 5% Donkey serum (DS) (Millipore) for 1 h. After blocking, cell cultures were incubated overnight at 4°C with primary antibodies diluted in PBS containing 1% of DS. After 3 washes with PBS, cells were incubated with secondary antibodies diluted in PBS containing 1% of DS for 1h at room temperature in the dark. The samples were washed with PBS three more times and incubated with 1.0 mg/mL 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. The following primary antibodies were used at the indicated dilutions: anti- SSEA4 (MC813-70) (mouse, 1:200; ThermoFisher, 41-4000), anti-Oct4 [EPR17929] - (Rabbit, 1:250; Abcam, ab181557), anti-Nestin [EPR17929] - (Rabbit, 1:500; BioLegend, 841901), anti-Tubulin β 3 (TUJ1) (mouse,1:1000; BioLegend, 801201), anti- MAP2 (Guinea pig, 1:1000; Synaptic systems, 188004), anti-phospho-Tau PHF-tau (Thr181) (mouse, 1:500; ThermoFisher, MN1050), Phospho-Fyn (Tyr530) (rabbit, 1:500; ThermoFisher, PA5-36644). The following secondary antibodies were used at the indicated dilutions: Alexa Fluor 488-conjugated Donkey anti-Mouse IgG IgG conjugated (1:400; ThermoFisher A-21202), Alexa Fluor 594- conjugated Donkey anti-rabbit IgG (1:400; ThermoFisher, A-31572), Alexa Fluor 594- conjugated Donkey anti-mouse IgG (1:400; ThermoFisher, A-21203), Alexa Fluor 647- conjugated Goat anti-Guinea Pig IgG (1:400; ThermoFisher, A-21450). All experiments

included cultures where the primary antibodies were not added, unspecific stain was not observed in such negative controls. Images were taken from the 63 × objective on a Leica TCS SP5 Confocal Laser Scanning Microscope coupled with the LAS AF lite software (Wetzlar, Germany). We used 386, 488 and 594 nm lasers, along with the appropriate excitation and emission filters. These settings were kept consistent while taking images from all cultures.

High-content screening (HCS)

NPC cells were plated at 1×104 cells per well on poly-L-ornithine/laminin-coated 96-well plates, after 3 days the cells were treated. After the treatment the cells were fixed and stained for pFYN kinase and Alexa Fluor™ 594 Phalloidin was used as a marker that defines the boundary of cells and DAPI for nuclear staining. A quantitative imaging analysis of the NPC cells was conducted through the Opera Phenix™ High Content Screening System at × 40 magnification using the Columbus™ Image analysis system. The following morphological features were assessed for both treated and control: number of cells and– number of Spot per cell. At least 15 fields were randomly selected and scanned per well of a 96-well plate in triplicate. To identify and remove any false readings generated by the system, three random Aβ and control wells were selected and counted manually (blind to group). For the p-Tau experiment, the treatment with compounds was done concomitantly with Aβ. Medium was changed after 3 days of treatment and cells were allowed to differentiate for 2 more days. On day 5, cells were fixed for immunocytochemistry. The morphological features assessed for both treatment and control were number of cells and Intensity Cell Alexa 568 per well.

Appendix

NMR Data

^{19}F NMR Individual Experiment

CHEMBL1302101

-64.63

-117.08

H2N

F

F

PBCHM13530069

CHEMBL1673279

−116.96

-110.0 -110.5 -111.0 -111.5 -112.0 -112.5 -113.0 -113.5 -114.0 -114.5 -115.0 -115.5 -116.0 -116.5 -117.0 -117.5 -118.0 -118.5 -119.0 -119.5 -120.0 -120.5 -121.0 -121.5 -122.0 -122.5 -123.0 -123.5 -124.0
f1 (ppm)

PBCHM4151894

−62.73

-47 -48 -49 -50 -51 -52 -53 -54 -55 -56 -57 -58 -59 -60 -61 -62 -63 -64 -65 -66 -67 -68 -69 -70 -71 -72 -73 -74 -75
f1 (ppm)

PBCHM4284201

−61.55

−61.26 −61.30 −61.34 −61.38 −61.42 −61.46 −61.50 −61.54 −61.58 −61.62 −61.66 −61.70 −61.74 −61.78 −61.82 −61.8
f1 (ppm)

CHEMBL1499171

−75.20

−75.05 −75.07 −75.09 −75.11 −75.13 −75.15 −75.17 −75.19 −75.21 −75.23 −75.25 −75.27 −75.29 −75.31 −75.33 −75.35 −75.37 −75.39 −75.41
f1 (ppm)

CHEMBL24458

-61.99

-60.6 -60.7 -60.8 -60.9 -61.0 -61.1 -61.2 -61.3 -61.4 -61.5 -61.6 -61.7 -61.8 -61.9 -62.0 -62.1 -62.2 -62.3 -62.4 -62.5 -62.6 -62.7 -62.8 -62.9 -63.0 -63.1 -63.2 -63.
f1 (ppm)

ZINC04653292

-74.13

-73.75 -73.80 -73.85 -73.90 -73.95 -74.00 -74.05 -74.10 -74.15 -74.20 -74.25 -74.30 -74.35 -74.40 -74.45 -74.50
f1 (ppm)

PBCHM4680099

-115.39

-113.5 -113.7 -113.9 -114.1 -114.3 -114.5 -114.7 -114.9 -115.1 -115.3 -115.5 -115.7 -115.9 -116.1 -116.3 -116.5 -116.7 -116.9 -117.1 -117.3
f1 (ppm)

PBCHM57487213

-62.27

-58.5 -59.0 -59.5 -60.0 -60.5 -61.0 -61.5 -62.0 -62.5 -63.0 -63.5 -64.0 -64.5 -65.0 -65.5
f1 (ppm)

CHEMBL1351172

−79.74

-75.5 -76.0 -76.5 -77.0 -77.5 -78.0 -78.5 -79.0 -79.5 -80.0 -80.5 -81.0 -81.5 -82.0 -82.5 -83.0 -83.5 -84.0
f1 (ppm)

PBCHM3049683

−62.64

-59.5 -60.0 -60.5 -61.0 -61.5 -62.0 -62.5 -63.0 -63.5 -64.0 -64.5 -65.0 -65.5
f1 (ppm)

PBCHM83397192

-60.60

60.44 -60.45 -60.46 -60.47 -60.48 -60.49 -60.50 -60.51 -60.52 -60.53 -60.54 -60.55 -60.56 -60.57 -60.58 -60.59 -60.60 -60.61 -60.62 -60.63 -60.64 -60.65 -60.66 -60.67 -60.68 -60.69 -60.70 -60.71 -60.72 -60.73 -60.74
f1 (ppm)

PBCHM120765

-62.25

-60.9 -61.0 -61.1 -61.2 -61.3 -61.4 -61.5 -61.6 -61.7 -61.8 -61.9 -62.0 -62.1 -62.2 -62.3 -62.4 -62.5 -62.6 -62.7 -62.8 -62.9 -63.0 -63.1 -63.2 -63.3 -63.4 -63.5 -63.6 -63.7 -63.8
f1 (ppm)

PBCHM2738092

-115.17

14.3 -114.4 -114.5 -114.6 -114.7 -114.8 -114.9 -115.0 -115.1 -115.2 -115.3 -115.4 -115.5 -115.6 -115.7 -115.8 -115.9 -116.0 -116.1 -116
f1 (ppm)

PBCHM609266

-73.20

-70.4 -70.6 -70.8 -71.0 -71.2 -71.4 -71.6 -71.8 -72.0 -72.2 -72.4 -72.6 -72.8 -73.0 -73.2 -73.4 -73.6 -73.8 -74.0 -74.2 -74.4 -74.6 -74.8 -75.0 -75.2 -75.4 -75.6 -75.
f1 (ppm)

PBCHM13486675

-63.42

-62.1 -62.2 -62.3 -62.4 -62.5 -62.6 -62.7 -62.8 -62.9 -63.0 -63.1 -63.2 -63.3 -63.4 -63.5 -63.6 -63.7 -63.8 -63.9 -64.0 -64.1 -64.2 -64.3 -64.4 -64.5 -64.6 -64.7 -64.8
f1 (ppm)

PBCHM33678746

-62.29

-61.80 -61.85 -61.90 -61.95 -62.00 -62.05 -62.10 -62.15 -62.20 -62.25 -62.30 -62.35 -62.40 -62.45 -62.50 -62.55 -62.60 -62.65 -62.70 -62.75 -62.80 -62.85 -62.90 -62.
f1 (ppm)

PBCHM69534497

−115.52

−108 −109 −110 −111 −112 −113 −114 −115 −116 −117 −118 −119 −120 −121 −122 −123
f1 (ppm)

ZINC20381469

−119.65

−119.10 −119.20 −119.30 −119.40 −119.50 −119.60 −119.70 −119.80 −119.90 −120.00 −120.10 −120.20 −120.30 −120.40 −120.5
f1 (ppm)

PBCHM11746172

57.89

PBCHM2782232

62.71

PBCHM81560982

−75.43

−66 −67 −68 −69 −70 −71 −72 −73 −74 −75 −76 −77 −78 −79 −80 −81 −82 −83 −84 −85
f1 (ppm)

PBCHM2732702

−116.87

−115.8 −115.9 −116.0 −116.1 −116.2 −116.3 −116.4 −116.5 −116.6 −116.7 −116.8 −116.9 −117.0 −117.1 −117.2 −117.3 −117.4 −117.5 −117.6 −117.7 −117
f1 (ppm)

¹⁹F NMR Scrambled Peptide Experiment

CHEMBL1673279

-116.97

116.55 -116.60 -116.65 -116.70 -116.75 -116.80 -116.85 -116.90 -116.95 -117.00 -117.05 -117.10 -117.15 -117.20 -117.25 -117.30 -117.35 -117.40 -117.45
f1 (ppm)

CHEMBL1499171

-75.21

-75.32

-75.04 -75.06 -75.08 -75.10 -75.12 -75.14 -75.16 -75.18 -75.20 -75.22 -75.24 -75.26 -75.28 -75.30 -75.32 -75.34 -75.36 -75.38 -75.40 -75.4
f1 (ppm)

CHEMBL1351172

PBCHM120765

PBCHM3049683

−62.64

−62.52 −62.53 −62.54 −62.55 −62.56 −62.57 −62.58 −62.59 −62.60 −62.61 −62.62 −62.63 −62.64 −62.65 −62.66 −62.67 −62.68 −62.69 −62.70 −62.71 −62.72 −62.73 −62.74 −62.75 −62.76 −62.77 −62.78 −62.79
f1 (ppm)

PBCHM4680099

−115.38

−112.6 −113.0 −113.4 −113.8 −114.2 −114.6 −115.0 −115.4 −115.8 −116.2 −116.6 −117.0 −117.4 −117.8 −118.2 −118.6 −119.0
f1 (ppm)

PBCHM57487213

-62.27

-61.90 -61.95 -62.00 -62.05 -62.10 -62.15 -62.20 -62.25 -62.30 -62.35 -62.40 -62.45 -62.50 -62.55 -62.60 -62.65 -62.70 -62.75
f1 (ppm)

PBCHM81560982

-75.44

-73.8 -74.0 -74.2 -74.4 -74.6 -74.8 -75.0 -75.2 -75.4 -75.6 -75.8 -76.0 -76.2 -76.4 -76.6 -76.8 -77.0 -77.2 -77.4 -77.6
f1 (ppm)

¹⁹F NMR Hit Expansion Competition Assay

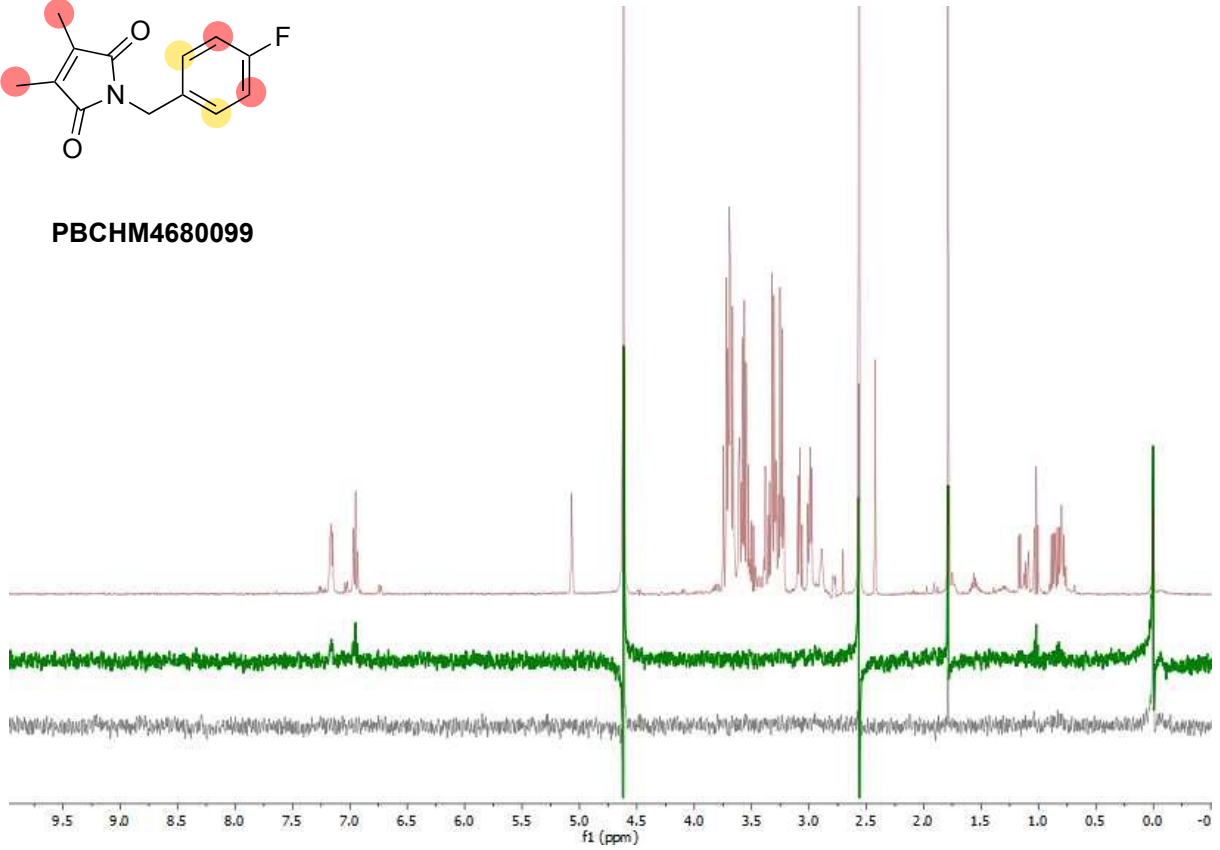**Seed Compound**
CHEMBL1673279

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PBCHM45210798

Seed Compound
PBCHM3049683

Analogue
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Seed Compound
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Analogue
ZINC00159801

Seed Compound
PBCHM3049683

Analogue
ZINC00057047

Seed Compound
PBCHM120765

Analogue
CHEMBL448523

-62.24

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f1 (ppm)

Seed Compound
PBCHM120765

Analogue
ZINC00120199

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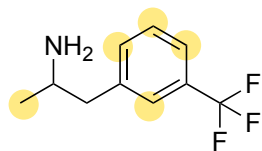
STD Experiment

CHEMBL1673279

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PBCHM4680099

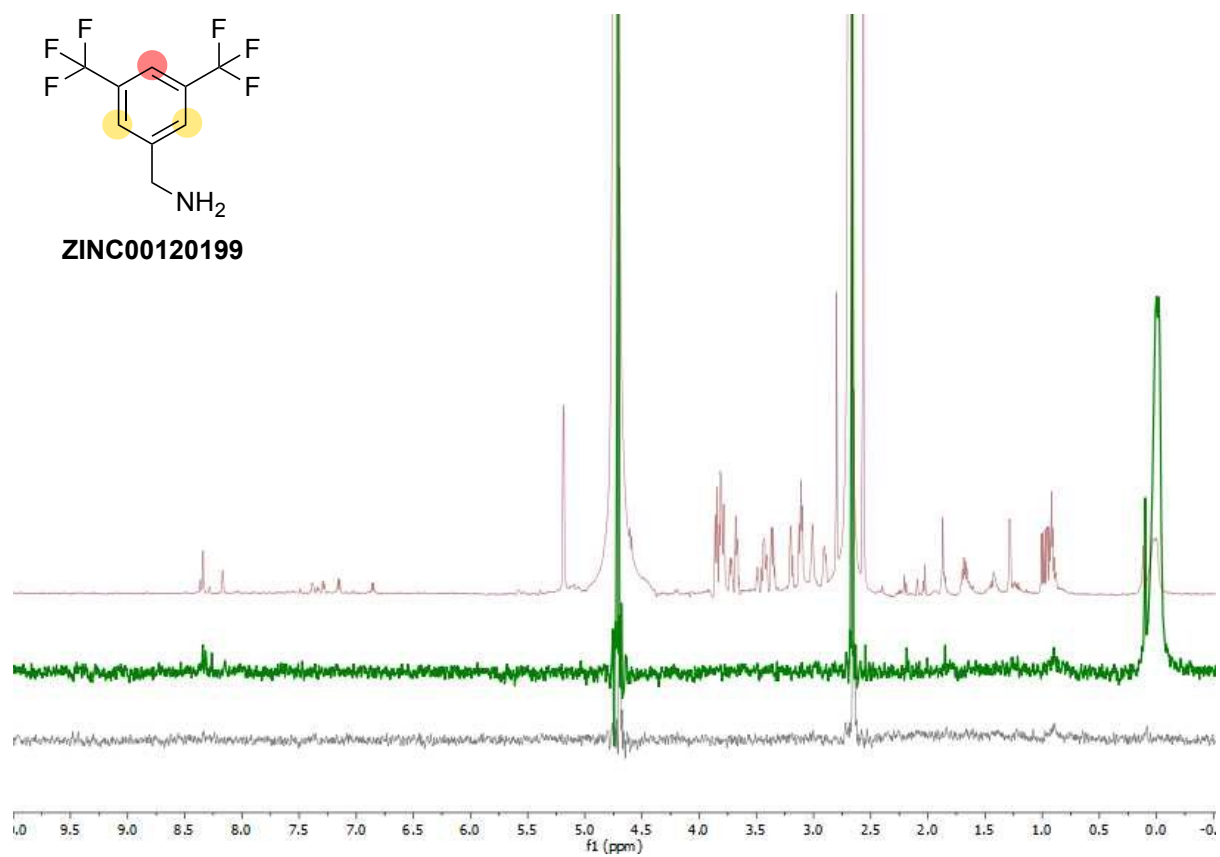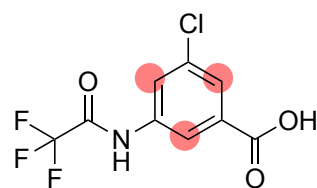**PBCHM57487213**
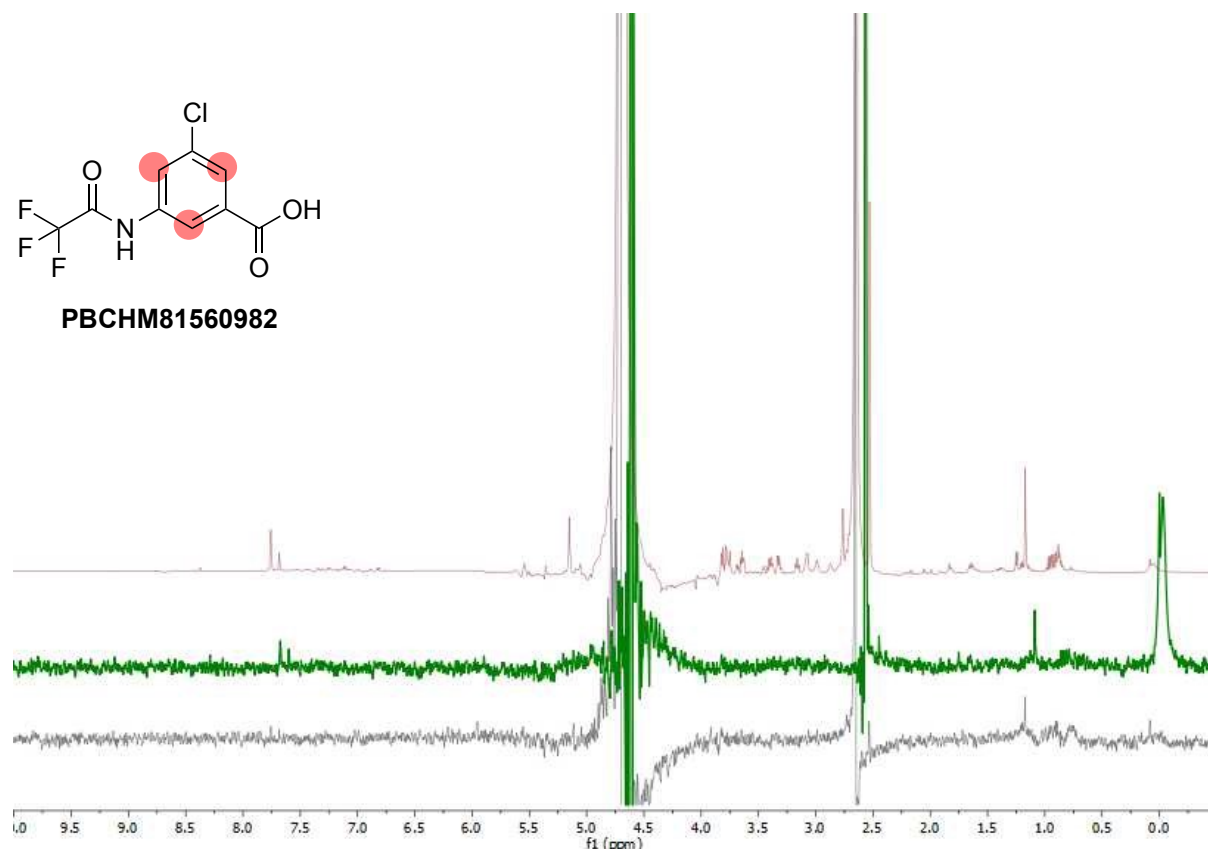
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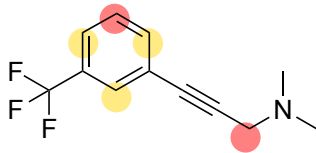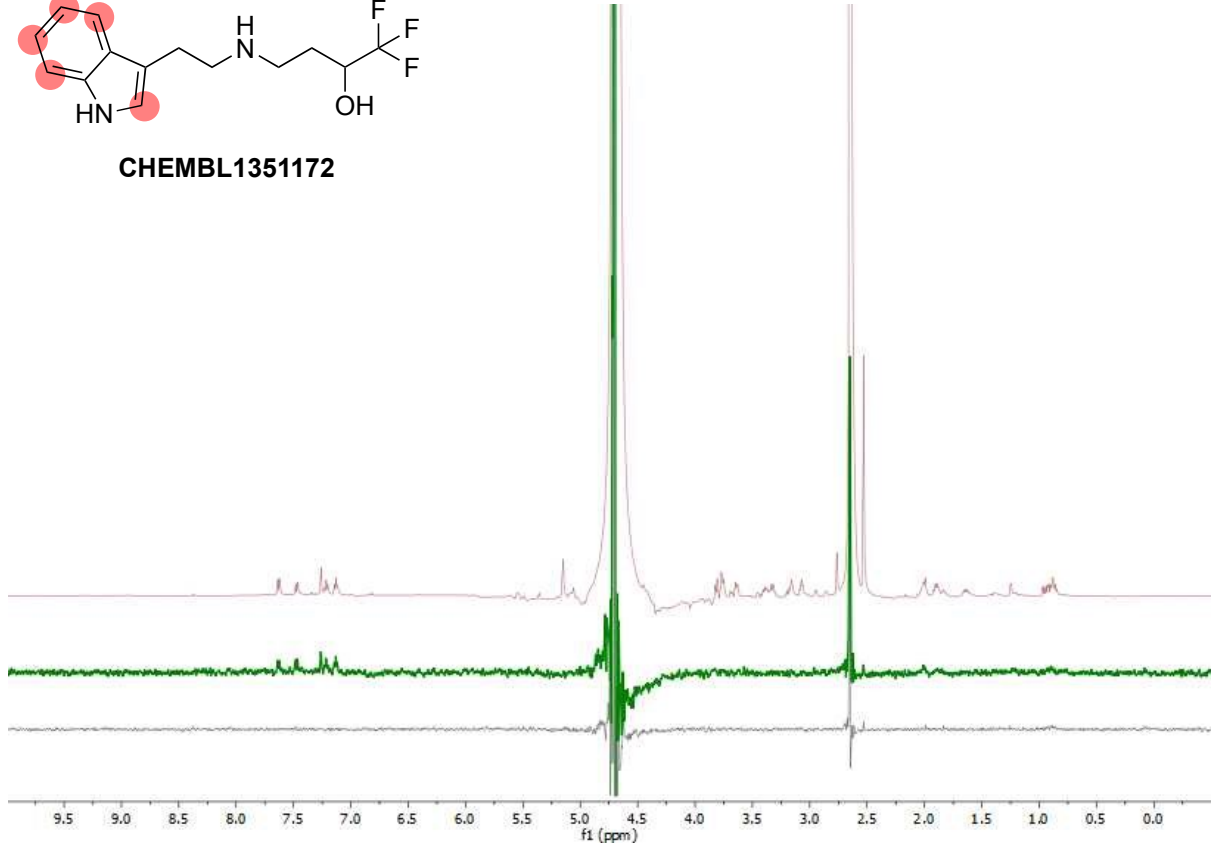
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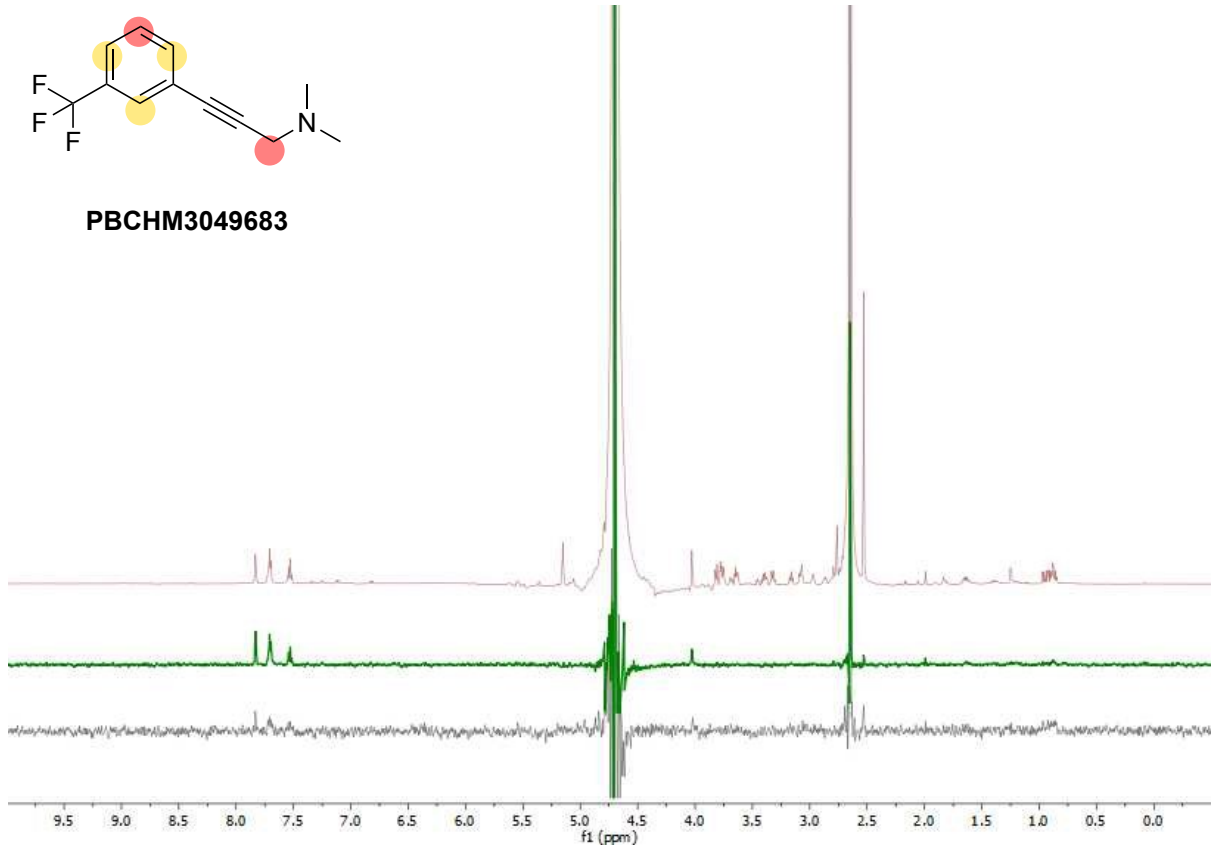
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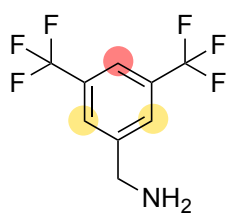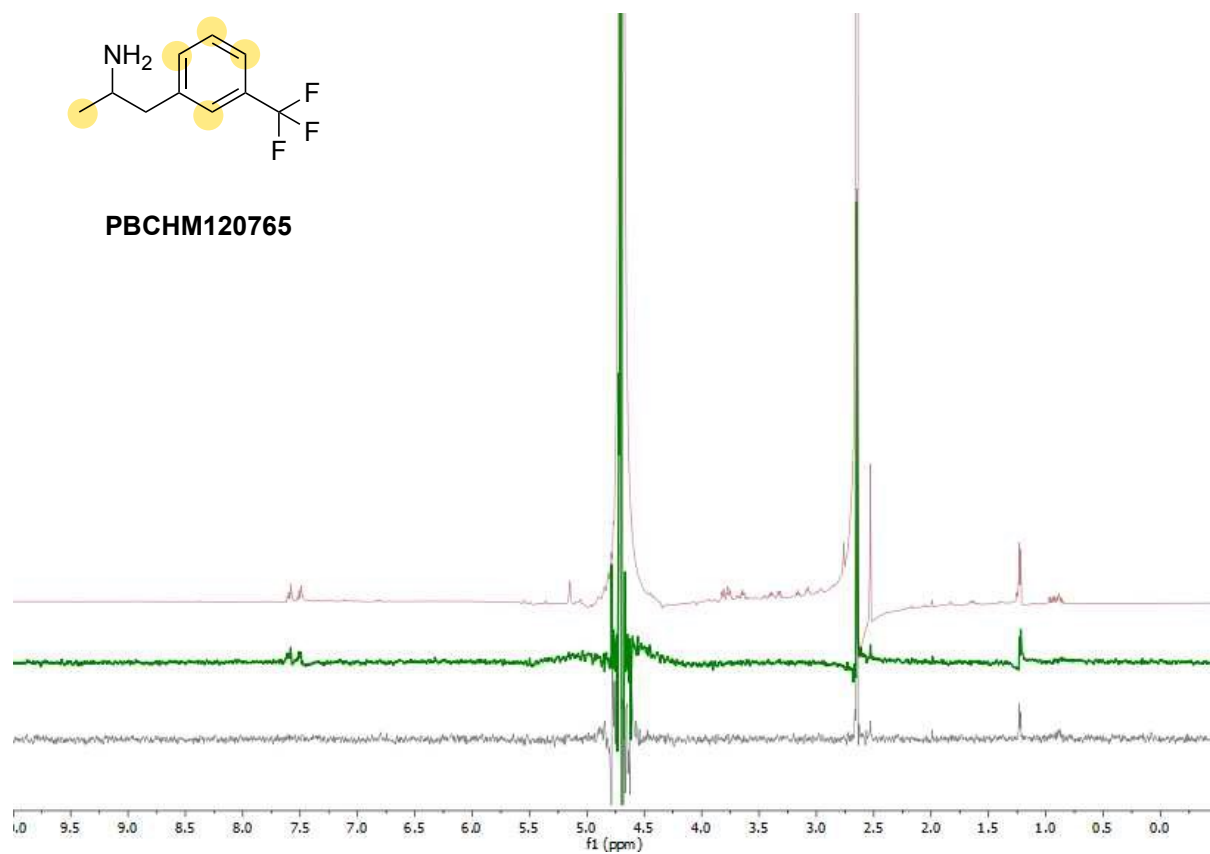
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PBCHM81560982

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