Supplementary Material: Do current therapeutic anti-Aβ antibodies for Alzheimer’s disease engage the target?

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Materials and methods

Antibody cloning and expression

Synthetic DNA for the heavy and light chains of each monoclonal antibody was synthesized by Genscript and subcloned into the pcDNA 3.1 vector (Invitrogen). DNA sequences were derived from published antibody amino acid sequences for bapineuzumab,[8] solanezumab,[9] and crenezumab.[10] N-terminal signal peptides for bapineuzumab and crenezumab were incorporated for the heavy chain (MGWSWIFLFLVSGTGGVLSE) and light chain (MESQTQVLMSLLFWVSGTCG) sequences. N-terminal signal peptides for solanezumab were incorporated for the heavy chain (MNFGGLSLIFLVLKLGVLC) and light chain (MKLPVRLLOMLFWIPASRC) sequences.

Heavy and light chain constructs were each cloned into the pcDNA 3.1 vector (Invitrogen). Plasmids were transformed into E. coli (DH5-alpha, Invitrogen) for amplification under ampicillin selection and purified with a PureLink™, HiPure Plasmid Megaprep Kit (Invitrogen) according to the manufacturer's instructions. Recombinant expression plasmids were then transfected into FreeStyle™ 293-F cells (Invitrogen) to allow expression of the recombinant antibodies.

Solanezumab expression

FreeStyle™ 293-F cells were cultured in FreeStyle expression medium (Invitrogen), and maintained at 37°C with an atmosphere of 8% v/v CO₂. Transient transfections were performed using 293Fectin transfection reagent (Invitrogen) according to the manufacturer's instructions. Cultures of 1000 mL were shaken in a Cellbag™ 2L (flow rate: 0.1-0.15 Lpm) on a bioreactor system. Cultures were supplemented with 0.1% Pluronic F68 (Invitrogen) and
0.5% Lucratone Lupin (Millipore) four and 24 hours post transfection, respectively, and the rocking angle adjusted to 9°C after 24 hours. Six days post-transfection the cell culture supernatants were harvested by centrifugation at 1000 x g.

**Bapineuzumab and Crenezumab expression**

293-F cells were cultured in FreeStyle expression medium (Invitrogen), and maintained at 37°C with an atmosphere of 8% v/v CO₂. The expression was performed in 4 L batches in a Certomat Ct plus incubator (Sartorius) by doing co-transfection of 1x10⁶ cells/mL with both DNA and 293Fectin transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cultures were supplemented with 5 mL/L of 10% v/v Pluronic F68 (Invitrogen), 5 mg/L Lucratone Lupin (Millipore) 4 hours post transfection, and 5mg/L of glucose 2 days post-transfection. The cell culture supernatants were harvested by centrifugation at 500 x g, and the media collected for purification.

**Antibody purification**

Harvested conditioned media was filtered through a 0.22 μm filter, concentrated to 200 mL by tangential flow filtration system (Millipore Proflux M12) and applied to a MabSelect SuRe HiTrap Protein A HP Column (5ml, Hitrap, GE Life Sciences, Sweden) previously equilibrated with PBS. Antibody was eluted with 0.1 M sodium citrate pH 3.5 and collected into 10% v/v final volume of 3.0 M Tris pH 8.0. Antibody was buffer exchanged into PBS using a HiLoad desalting column 26/10 (GE Life Sciences, Sweden) and concentrated to 1 mg/mL with a centrifugal concentrator (Amicon ultra, 50 kDa MWCO) for storage at -80°C.
**Fab production**

Corresponding antigen binding fragments for each antibody was produced with the respective heavy chain clone modified to express a hexa-histidine sequence in place of the Fc portion. Expression was identical to that described by Miles et. al [5] for Bapineuzumab.

For each Fab, 4 L of harvested media was concentrated to 200 mL by tangential flow filtration system (Millipore Proflux M12). The concentrated media was centrifuged at 20000 x g for 30 minutes before being purified by immobilized-metal affinity chromatography. The supernatant containing Fab was incubated for 1 hour with equilibrated Ni-NTA affinity resin (Qiagen). The mixture was washed 4 times with 20 mM Tris pH 8.0, 150 mM NaCl, and 20 mM imidazole. The protein of interest was eluted with 20 mM Tris pH 8.0, 150 mM NaCl, and 500 mM imidazole. Eluted sample was further purified by size exclusion chromatography with a HiLoad Superdex 200 20/60 run in PBS on an AKTApurifier (GE Healthcare). Fractions were concentrated to 2 mg/mL with a centrifugal concentrator (Amicon ultra, 10 kDa MWCO). Purification steps were monitored by SDS-PAGE.

**Antibody recognition of synthetic Aβ species**

Aβ binding activity of the recombinant antibodies were tested by surface plasmon resonance (SPR) on a ProteOn XPR36 system (Bio-Rad). Aβ1-40, with an additional C-terminal biotinylated lysine residue (Anaspec) was dissolved in DMSO, diluted 1000-fold in PBS and conjugated to an NLH sensor chip. Of the six ligand channels available, four were liganded with biotinylated Aβ at very high (~2000 r.u.), medium and very low surface density (~1 r.u.). A flow rate of 30 μL/min was used and binding was monitored at 25°C. Initial kinetic experiments were performed in PBS, pH 7.4. In these experiments, no binding was observed
for bapineuzumab at an antibody concentration of 30 μM. Tween-20 was titrated into the reaction buffer and binding response curves improved for all the antibodies, but the improvement in bapineuzumab binding was stark. Bapineuzumab was then injected at 30 μM at five concentrations of Tween-20 (0.02, 0.01, 0.005, 0.001, 0.0005% (v/v)) and a PBS blank; however, no improvement in binding was observed for Tween-20 concentrations above 0.005% (v/v) and so subsequent experiments were performed using this concentration. Surfaces were regenerated after each experiment with 0.85% (v/v) phosphoric acid followed by 25 mM NaOH. Antibodies were diluted from stock solutions with starting concentrations of 2.9, 7.0 and 2.8 μM for bapineuzumab, solanezumab, and crenezumab, respectively, and at four other concentrations in a series of 10-fold dilutions. Response curves were collected in duplicate and for the range of ligand surface densities. A 300 sec association phase was followed by a 2400 sec (40 min) dissociation phase.

Parallel kinetic experiments were performed to test the ability of each monoclonal antibody to bind truncated and modified synthetic Aβ peptides, exploiting the 6 x 6 channel configuration of the ProteOn XPR36 system. Bapineuzumab, solanezumab, and crenezumab were conjugated directly to a GLH sensor chip by amine coupling (10 mM sodium phosphate, pH 5.5) and at high surface density. All measurements were performed in PBS with 0.005% (v/v) Tween-20, pH 7.4, and at 25 °C. Surfaces were regenerated after each experiment with 0.85% (v/v) phosphoric acid followed by 25 mM NaOH. A flow rate of 100 μL/min, per channel, was used throughout these experiments.

The Aβ peptides screened included Aβ1-28, Aβ1-40, Aβ1-42, the N-terminally truncated peptides Aβ3-42, Aβ4-42, and the truncated and modified pEAβ3-42, and pEAβ1-142. Aβ1-28, Aβ1-40, Aβ1-42 were purchased from the Keck laboratory, Yale. All other Aβ peptides were made in house.
using published methods.[3] These peptides exhibit varied complex solution properties, including propensity to bind metals, aggregate and form higher oligomeric structures. In addition, the structure of the epitopes recognized by each of these antibodies, with the exception of bapineuzumab,[5] was not known. The heterogeneous nature of the analytes and potential variations in the distribution of antibody recognition epitopes made it difficult to establish the effective concentration of each analyte. As such, a comprehensive kinetic evaluation was not attempted and equilibrium coefficients were omitted for less soluble peptides where apparent $k_a$ values varied dramatically with peptide concentration and time due to aggregation. Peptides were dissolved in NaOH and dissolved to circa 0.5 mg/ml total peptide from powder. Each analyte was passed over the antibody ligands at five concentrations in a series of 3-fold dilutions plus a buffer control, and duplicated, soon after dissolution and centrifugation (13,000 x g). The strength of each antibody-peptide interaction was assessed by monitoring dissociation of the complexes over a 40 min period following each association phase.

**Collection and preparation of transgenic mouse brain samples**

Cortical samples collected from 15 month old female APP\textsubscript{SWE}tg mice (Tg2576) were homogenized following removal of the cerebellum, olfactory bulbs, medulla, and pons. The isolated tissue was added to 1 x Dulbecco phosphate buffered saline (PBS), devoid of Mg and Ca (Invitrogen), but containing ethylenediaminetetraacetic acid (EDTA) free protease inhibitors (Roche) to yield 100 mg tissue per mL of buffer. The brain sample was then homogenized, using an ultrasonic cell disrupter (Virsonic 600; VirTis, Gardiner, NY), three times for 30 sec each at 24,000 rpm. The protein concentrations of the samples were assessed
using the Bradford Assay (BioRad) before the homogenates were aliquoted and stored at -80 °C until required. All sample preparation was undertaken on ice.

Forty (40) μL of brain homogenate was combined with 40μL of 8 M urea and 240 μL of 0.5% Triton-X 100 in PBS. This mixture was sonicated in an ultra-sound bath for 10 min. All steps were performed on ice.

**Collection and preparation of transgenic mouse blood samples**

Samples were collected from 9 month old female APPSweTg mice (Tg2576). Blood was withdrawn from anaesthetized mice via cardiac puncture into pre-labelled and pre-chilled microtainer tubes containing EDTA (K2); approximately 500-1000 µL of blood was collected from each animal. Samples were centrifuged for 20 min at 4 °C at 1900 x g before the upper plasma layer was removed leaving the cellular fractions.

Forty (40) μL of plasma was combined with 40μL of 8 M urea and 480 μL of 0.5% Triton-X 100 in PBS. This mixture was sonicated in an ultra-sound bath for 10 min. All steps were performed on ice.

**Collection, characterization, and preparation of human cortical tissue**

Human brain tissue was collected at autopsy. The sourcing and preparation of the tissue was conducted by the Australian Brain Bank Network (ABBN). Pathological diagnosis of AD was made in accordance with the standard criteria set out by the National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological
Assessment of Alzheimer’s Disease.[1,7] All procedures were conducted in accordance with the Florey Neurosciences Ethics Committee.

Grey matter was isolated from the post-mortem frontal cortices of sporadic AD affected individuals. The isolated tissue was subsequently added to 1 x Dulbecco phosphate buffered saline (PBS), devoid of Mg and Ca (Invitrogen), but containing ethylenediaminetetraacetic acid (EDTA) free protease inhibitors (Roche) to yield 100 mg tissue per mL of buffer. The brain sample was then homogenized, using an ultrasonic cell disrupter (Virsonic 600; VirTis, Gardiner, NY), three times for 30 sec each at 24,000 rpm. The protein concentrations of the samples were assessed using the Bradford Assay (BioRad) before the homogenates were aliquoted and stored at -80 °C until required. All sample preparation was undertaken on ice.

Brain homogenates (40 µL) were prepared with 40 µL of 8 M urea and 240 µL of wash-buffer and placed for 10 min in an ultra-sound bath with ice. All steps were performed on ice.

**Collection, characterization, and preparation of human blood samples**

Blood samples were collected from AD patients enrolled in the Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Aging.[2] AD patients met the criteria for probable AD in accordance with the NINCDS-ARDA[4] (for full details see [2]).

Venesection was used to collect 4 mL samples of whole blood in EDTA vacutainers 1.6 mg/mL of blood (C_{EDTA}, Greiner Bio-One) following overnight fasting. Blood processing commenced within 20 min of sample acquisition. The samples were centrifuged for 20 min at 4 °C at 1900 x g before the upper plasma layer was removed. The buffy coat layer below the plasma layer was removed before being combined with 250 µL PBS. This mixture was then
added to 5 mL Ficoll-Paque PLUS (GE life Sciences) and centrifuged for 20 min at 20 °C at 400 x g with soft break on. The supernatant was removed and added to 7 mL PBS before it was centrifuged for 5 min at 20 °C at 1900 x g. The resulting supernatant was discarded and the pellet was resuspended in 500 µL Milli-Q H₂O. This white blood cell / cellular fraction was aliquoted and stored in low binding polypropylene tubes at -80 °C until required. All samples underwent a single freeze / thaw cycle.

Forty (40) µL of blood, either plasma or cellular fraction was combined with 40 µL of 8 M urea (Merck; Kilsyth, Victoria) and 480 µL of 0.5% Triton-X 100 (Labchem) in PBS. This mixture was sonicated in an ultra-sound bath for 10 min. All steps were performed on ice.

For spiked plasma samples, 40 µL of plasma was combined with 40 µL of 8 M urea (Merck; Kilsyth, Victoria) and 480 µL of 0.5% Triton-X 100 (Labchem) in PBS. Synthetic Aβ₁₋₄₂ (Keck Laboratory) was added to form a final concentration of 50 pM. This mixture was sonicated in an ultra-sound bath for 10 min. All steps were performed on ice.

**SELDI-TOF MS analysis of biological samples**

Mass spectrometric analysis of the human samples was carried out using ProteinChip® PS10 Arrays (Bio-Rad; CAT #C55-30044). Arrays were loaded with 2 µL of bapineuzumab, crenezumab, solanezumab, WO2, or 4G8 at a concentration of 0.25 mg/mL. Like bapineuzumab, WO2 binds to the N-terminus of Aβ at residues 2-8 and was obtained from the WEHI Monoclonal Antibody Facility. 4G8, like crenezumab and solanezumab, targets the mid-region of the Aβ peptide binding to residues 17-24 and was purchased from Covance (SIG-39220). Chips were then incubated for two hours at 20 °C in a humidity chamber before excess antibodies were removed and 10 µL blocking buffer (0.5 M ethanolamine in PBS) was
added and incubated for 30 min. After the removal of the blocking buffer, arrays were washed three times for five min with 120 µL of 0.5% Triton X-100/PBS (wash-buffer) followed by three five min washes with 100 µL PBS.

For human samples and spiked human samples, 150 µL of sample mix was added to each spot and incubated at room temperature for 90 min. For mouse samples, 120 µL of sample mix was added under the aforementioned conditions.

Subsequent to incubation, samples were removed before the arrays underwent three 10 second washes on a vigorous shaking table with 150 µL wash-buffer, and 150 µL PBS, followed by two washes with 150 µL HEPES. The arrays were then dried before two 1 µL aliquots of a 50% saturated solution of SPA EAM were applied, with air-drying between treatments. The 50% saturated solution was prepared by suspending 5 mg ProteinChip SPA EAM (Bio-Rad; CAT # C30-00002) in 0.5% trifluoroacetic acid (TFA; Sigma-Aldrich; St Louis, Missouri), 50% acetonitrile (ACN; High performance liquid chromatography (HPLC) grade), 15% isopropyl alcohol (IPA; HPLC grade), and 34.5% HPLC grade H₂O.

Each sample was analyzed in duplicate using a ProteinChip SELDI System Enterprise Edition (BioRad). All spectra were internally normalized and peak intensities were normalized using total ion current. Aβ species, including potential oxidations, were matched to peaks within the resulting spectra using M/Z.

**Immunoprecipitation in conjunction with LC/MSMS**

IP assays were performed using recombinantly purified hexa-histidine tagged FAB fragments. Briefly 100 µg of FAB fragments in PBS were incubated with magnetic Ni beads
(Sigma) for 2 hours. The beads were then washed 4 times in PBS with 10 mM imidazole. Together with Ni resin without any FABs bound, they were then incubated with 50 μL of human plasma overnight at 4 °C. The resins were then washed thoroughly in PBS with 10 mM imidazole. The resin was then resuspended in 30 μL of 25 mM triethylammonium bicarbonate (TEAB), with 10 μg/mL sequencing grade trypsin (Sigma-Aldrich) and incubated overnight at 37 °C.

LC-MSMS was carried out on a LTQ Orbitrap Elite (Thermo Scientific) with a nanoelectrospray interface coupled to an Ultimate 3000 RSLC nanosystem (Dionex). The nanoLC system was equipped with an Acclaim Pepmap nano-trap column (Dionex – C18, 100 Å, 75 μm x 2 cm) and an Acclaim Pepmap analytical column (Dionex C18, 2μm, 100 Å, 75 μm x 15 cm). 5 μl of the peptide mix was loaded onto the trap column at an isocratic flow of 4 μl/min of 3% CH₃CN containing 0.1% formic acid before the enrichment column was switched in-line with the analytical column. The eluents used for the liquid chromatography were 0.1% (v/v) formic acid (solvent A) and 100% CH₃CN/0.1% formic acid (v/v). 0.1% formic acid (v/v). The flow following gradient was used : 3% to 12% B for 3 min, 12% to 35% B in 60 min, 35% to 80% B in 13 min and maintained at 80% B for 6 min followed by equilibration at 3% B for 10 min before the next sample injection. The LTQ Orbitrap Elite mass spectrometer was operated in the data dependent mode with nano ESI spray voltage of +2.0 kv, capillary temperature of 250°C and S-lens RF value of 60%. A data dependent mode whereby spectra were acquired first in positive mode with full scan scanning from m/z 300-1650 in the FT mode at 240,000 resolution followed by Collision induced dissociation (CID) in the linear ion trap with ten most intense peptide ions with charge states ≥2 isolated and fragmented using normalized collision energy of 35 and activation Q of 0.25.
Acquired MS/MS data were searched against all human proteins in UniProt using Mascot. The false discovery rate was set to 2.5%. Protein hits were then filtered against those identified in the Ni resin without any FAB. Protein hits for crezenumab and solezumab were subsequently filtered against those for bapineuzemab, and screened for unique proteins identified in both samples.
### Results

**Supplementary Table 1 Comparisons between the Complementarity Determining Regions of solanezumab and crenezumab**

Amino acid sequences of the Complementarity Determining Regions (CDRs) from the heavy (H1, H2, and H3) and light (L1, L2, and L3) chains of the immunotherapeutic antibodies solanezumab and crenezumab. The conservative differences are shown in italics and the five locations where non-conservative differences occur are underlined and bold.

<table>
<thead>
<tr>
<th>Light Chains</th>
<th>Heavy Chains</th>
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<tr>
<td>L1</td>
<td>L2</td>
</tr>
<tr>
<td>Solanezumab</td>
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<tr>
<td>Crenezumab</td>
<td>RSSQL/YSQ/S/LYTLYLH</td>
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Supplementary Table 2 Summary of the affinity constants ($k_a$), dissociation rate constants ($k_d$) and the dissociation constants ($K_D$) for bapineuzumab, crenezumab and solanezumab and synthetic Aβ species

<table>
<thead>
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<th>Bapineuzumab</th>
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<tr>
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n = 5

^a n = 15
Supplementary Table 3 Observed MS/MS fragmentation of a C-terminal APP peptide pulled down by a bapineuzumab IP

K.KQYTSIHHGVVEVDAAVTPEER.H (residues 726 – 747; measured mass 2464.2412 Da)

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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>y*++</td>
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Supplementary Fig. 1 Spectral profile of synthetic Aβ1-42 in human plasma using bapineuzumab, crenezumab, and solanezumab

Representative SELDI-TOF MS spectra of AD-affected plasma spiked with 50 pM synthetic Aβ1-42 arising from analysis with bapineuzumab, crenezumab, and solanezumab in addition to the laboratory standards, WO2 and 4G8
References

1 Consensus recommendations for the postmortem diagnosis of alzheimer's disease. The national institute on aging, and reagan institute working group on diagnostic criteria for the neuropathological assessment of alzheimer's disease. Neurobiology of aging 18: S1-2


