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ORIGINAL PAPER

# Do current therapeutic anti-Aβ antibodies for Alzheimer's disease engage the target?

**Andrew D. Watt · Gabriela A. N. Crespi · Russell A. Down · David B. Ascher · Adam Gunn · Keyla A. Perez · Catriona A. McLean · Victor L. Villemagne · Michael W. Parker · Kevin J. Barnham · Luke A. Miles**

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**Abstract** Reducing amyloid-β peptide (Aβ) burden at the pre-symptomatic stages of Alzheimer's disease (AD) is currently the advocated clinical strategy for treating this disease. The most developed method for targeting Aβ is the use of monoclonal antibodies including bapineuzumab, solanezumab and crenezumab. We have synthesized these antibodies and used surface plasmon resonance (SPR) and mass spectrometry to characterize and compare the ability of these antibodies to target Aβ in transgenic mouse tissue as well as human AD tissue. SPR analysis showed that the antibodies were able to bind Aβ with high affinity. All of the antibodies were able to bind Aβ in mouse tissue. However, significant differences were observed in human brain tissue. While bapineuzumab was able to capture a variety of N-terminally truncated Aβ species, the Aβ detected using solanezumab was barely above detection limits while crenezumab did not detect any Aβ. None of the antibodies were able to detect any Aβ species in human blood. Immunoprecipitation experiments using plasma from AD subjects showed that both solanezumab and crenezumab have extensive cross-reactivity with non-Aβ related proteins. Bapineuzumab demonstrated target engagement with brain Aβ, consistent with published clinical data. Solanezumab and crenezumab did not, most likely as a result of a lack of specificity due to cross-reactivity with other proteins

K. J. Barnham and L. A. Miles contributed equally to this work.

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A. D. Watt · R. A. Down · A. Gunn · K. A. Perez · C. A. McLean · V. L. Villemagne · K. J. Barnham
Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia

A. D. Watt · R. A. Down
Department of Pathology, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia

A. D. Watt · R. A. Down · A. Gunn · K. A. Perez · K. J. Barnham
Neuroproteomics Platform, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia

G. A. N. Crespi · D. B. Ascher · M. W. Parker · L. A. Miles
ACRF Rational Drug Discovery Centre and Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, Fitzroy, VIC 3056, Australia

C. A. McLean
Department of Anatomical Pathology, The Alfred Hospital, Melbourne, VIC 3084, Australia

V. L. Villemagne
Department of Medicine, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia

V. L. Villemagne
Department of Nuclear Medicine and Centre for PET, Austin Health, Heidelberg, VIC 3084, Australia

M. W. Parker · L. A. Miles (✉)
Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia
e-mail: lmiles@svi.edu.au

K. J. Barnham (✉)
Department of Pharmacology, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia
e-mail: kbarnham@unimelb.edu.au

containing epitope overlap. This lack of target engagement raises questions as to whether solanezumab and crenezumab are suitable drug candidates for the preventative clinical trials for AD.

**Keywords** Alzheimer's disease · β-Amyloid · Bapineuzumab · Crenezumab · Solanezumab · Mass spectrometry

## Introduction

If the β-amyloid (Aβ) hypothesis of Alzheimer's disease (AD) is correct [1, 2], then the aim of any therapeutic intervention should be to remove toxic Aβ peptides from the brain. In attempts to achieve this, billions of dollars have been spent on clinical trials of anti-Aβ therapies for treatment of mild to moderate sufferers of AD. Now, after unmitigated trial failures in symptomatic patients, the field is looking for a therapeutic window earlier in the disease process where Aβ-directed interventions aim to prevent downstream pathology thereby delaying or stopping the development of dementia before significant damage to the brain has occurred.

Anti-Aβ antibodies are the most developed potential therapeutics for AD. Bapineuzumab (Pfizer, Johnson & Johnson) derives specificity for Aβ by binding it in a conformationally dependent manner, recognizing the five extreme N-terminal residues of Aβ as a helix with the N-terminus buried in the antibody surface [3]. Bapineuzumab is the only antibody clinically reported to reduce brain amyloid burden as well as decreasing cerebrospinal fluid (CSF) levels of both total Tau (t-Tau) and phosphorylated-Tau (p-Tau) in mild to moderate AD patients [4, 5]. However, despite successful target engagement, a large-scale Phase 3 trial of bapineuzumab to treat mild to moderate AD was prematurely halted when high doses were found to promote vasogenic oedema and other amyloid-related imaging abnormalities [6].

Solanezumab (Eli Lilly) targets the central region of Aβ and is reported to selectively bind to soluble monomeric Aβ with little affinity for oligomeric/fibrillar forms [7, 8]. On examination of transgenic mice expressing Aβ solely within the central nervous system (CNS), it was hypothesized that solanezumab acts as an Aβ sink in the periphery rather than in the CNS [9, 10], disrupting the equilibrium between plasma and brain Aβ, thereby driving amyloid from the brain into blood. Large Phase 3 clinical trials showed no evidence that administration of solanezumab shifted brain amyloid burden or downstream biological markers of disease. Post hoc analysis indicated that treatment with solanezumab slowed cognitive decline in patients with mild, but not moderate, forms of the disease. This significant post hoc finding coupled with solanezumab's safety profile led to the compound being recommended as the first therapeutic agent to be assessed in the anti-amyloid treatment in asymptomatic Alzheimer's disease (A4) prevention trial [11].

Crenezumab, like solanezumab, binds the linear central portion of Aβ. It has been reported that crenezumab binds preferentially to Aβ fibrils and oligomers over monomeric species, and reduces amyloid plaque burden in transgenic mice [12]. However, despite crenezumab's reported affinity for oligomeric Aβ, it is important to note that there are currently no techniques with the capacity to monitor the in vivo burden of these putatively toxic species. Crenezumab is built on an IgG4 backbone, unlike bapineuzumab and solanezumab (both are IgG1). The IgG4 backbone enables the drug to mildly stimulate microglia enough for Aβ uptake but not to evoke an inflammatory response compared to the same drug on an IgG1 backbone and can therefore be administered at higher doses [12]. Crenezumab has recently been selected for evaluation in the Alzheimer's Prevention Initiative's trial in a presymptomatic Colombian kindred with autosomal-dominant AD [13].

To date, direct comparisons between these antibodies have been limited, particularly with regard to their respective Aβ binding profiles. Therefore, the aim of the current investigation was to provide further characterization of the Aβ binding profiles of bapineuzumab, crenezumab, and solanezumab using synthetic Aβ peptides and surface plasmon resonance (SPR) before analyzing their binding profiles in both transgenic mouse and AD-affected tissues alongside comparable commercially available antibodies (WO2 and 4G8) using surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF MS).

## Methods

Comprehensive methodological descriptions are provided in Supplementary Material. Therapeutic antibodies and their Fabs were expressed in FreeStyle™ 293-F cells (Invitrogen), purified to homogeneity and stored in PBS in 20 μL aliquots until required. pcDNA 3.1 vector (Invitrogen) constructs encoding each antibody chain were derived from synthetic DNA (Genscript) corresponding to published amino acid sequences: [14, 15] Bapineuzumab (Patent US 20080292625 A1) [16]; solanezumab (Patent WO 2001062801 A2, CAS 955085-14-0, CHEMBL1743072) [17]; crenezumab (Patent EP 2574345 A1, CAS 1095207-05-8, CHEMBL1743004) [18]. Murine antibody WO2 was obtained from the WEHI Monoclonal Antibody Facility and murine 4G8 was purchased from Covance (SIG-39220).

Aβ binding activity of the recombinant antibodies was tested by surface plasmon resonance (SPR) in parallel on a ProteOn XPR36 system (Bio-Rad). The Aβ peptides screened included $Aβ_{1–28}$, $Aβ_{1–40}$, $Aβ_{1–42}$, the N-terminally truncated peptides $Aβ_{3–42}$, $Aβ_{4–42}$, and the truncated and modified $pEAβ_{3–42}$, and $pEAβ_{11–42}$. $Aβ_{1–28}$, $Aβ_{1–40}$, $Aβ_{1–42}$ were purchased from the Keck laboratory, Yale. All other Aβ peptides were made in-house using published methods [19].

Cortical samples were collected from 15-month-old female $APP_{SWE}$tg mice (Tg2576) and homogenized, while mouse blood samples were collected from 9-month-old female $APP_{SWE}$tg mice (Tg2576). Samples were stored at −80 °C.

Human brain tissue collected at autopsy and characterized by the Australian Brain Bank Network (ABBN) and preparation of the cortical homogenates was in accordance with previously reported methodologies [20]. Blood samples were collected from AD patients enrolled in the Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Aging [21]. All procedures were conducted in accordance with the Florey Neurosciences Ethics Committee.

Mass spectrometric analysis was carried out using ProteinChip® PS10 Arrays (Bio-Rad; CAT #C55-30044) using previously published protocols [20, 22].

6His-tagged Fabs for each therapeutic antibody were coupled to magnetic Nickel beads (Sigma) for immunoprecipitation in AD plasma according to the manufacturer's instructions. Biomolecules pulled down by the antibodies were proteolytically digested and detected by liquid chromatography (LC)-MS/MS on a LTQ Orbitrap Elite (Thermo Scientific). Acquired MS/MS data were searched against all human proteins in UniProt using Mascot [23, 24].

## Results

### Crenezumab has strong sequence similarities with solanezumab

While crenezumab has an IgG4 backbone, solanezumab has an IgG1 backbone; however, the complementarity determining regions (CDRs) are identical in length, with no insertions or deletions (see Supplementary Table 1). Three CDRs are of identical sequence (L2, L3, and the hypervariable H3). Besides a few very conservative amino acid substitutions, there are merely five non-conservative amino acid differences in total between the CDRs of solanezumab and crenezumab. CDR 2 of the heavy chain in solanezumab can be glycosylated at N56 on the heavy chain that has the potential to sterically hinder Aβ affinity.

### Antibodies bind synthetic Aβ species with high affinity

Bapineuzumab, crenezumab, and solanezumab preparations studied here were expressed in human embryonic kidney cells, which might be different to the methods used for production of the clinically tested recombinant humanized samples and their murine equivalents studied in animal models. The clinical antibodies reportedly have a single N-linked glycosylation site in their respective Fc portions that mediates Fc receptor binding rather than ligand affinity and specificity of the complementarity determining regions conserved in humanized and murine equivalent antibodies. There is a single glycosylation site in the Fv domain of solanezumab: N56 of the heavy chain CDR2 that can sterically modify affinity for Aβ, from a $K_D$ of 4 pM for fully glycosylated antibody to a $K_D$ of 0.8 pM for the N56S/N56T mutated (unglycosylated) antibody (patent WO/2003/016466). This demonstrates that solanezumab retains extremely high affinity for Aβ regardless of differential glycosylation at this site; which might arise from differential expression systems for antibody production. We have used one of the preferred mammalian expression systems prescribed in each antibody patent to minimize the possibility of differential glycosylation. We initially performed SPR experiments to ensure that these antibodies were able to bind a range of synthetic Aβ peptides in accordance with their reported epitopes [16–18]. Mean rate association constants ($k_a$) for bapineuzumab, solanezumab, and crenezumab with captured Aβ as determined by SPR were found to be 7, 0.8 and $6 \times 10^3$ $M^{-1}$ $s^{-1}$, respectively (Fig. 1a). Dissociation curves were flat and derived dissociation constant ($k_d$) values were in the order of $1 \times 10^{-5}$ $s^{-1}$ or lower (Supplementary Table 2). Drifting baselines, most likely due to Aβ aggregation, hampered efforts to resolve accurate $K_D$ values. However, we established that the antibodies bapineuzumab, solanezumab and crenezumab recognized synthetic $Aβ_{1–40}$ with binding constants in the low nanomolar and sub-nanomolar ranges when Aβ is conjugated to the chip (Fig. 1a). With antibodies coupled to the SPR chip and Aβ peptides introduced as analytes, the flat dissociation phase response curves for solanezumab persisted, yielding unreliable $k_d$ values down to $10^{-17}$ $s^{-1}$, and a mean $k_d$ value in the order of $10^{-6}$ $s^{-1}$, i.e., beyond the limits of detection. Apparent $k_d$ values increased for bapineuzumab and crenezumab which were reproducible and in the order of $10^{-4}$ $s^{-1}$ and $10^{-3}$ $s^{-1}$, respectively. Apparent $K_D$ values for soluble Aβ species binding to bapineuzumab and crenezumab are in the order of low nanomolar, whereas apparent $K_D$ values for solanezumab binding are in the picomolar or better range (Supplementary Table 2). Bapineuzumab did not recognize Aβ pre-treated with NaOH/HFIP in PBS, but demonstrated strong binding as reported above in PBS plus 0.005 % (v/v) Tween-20. This

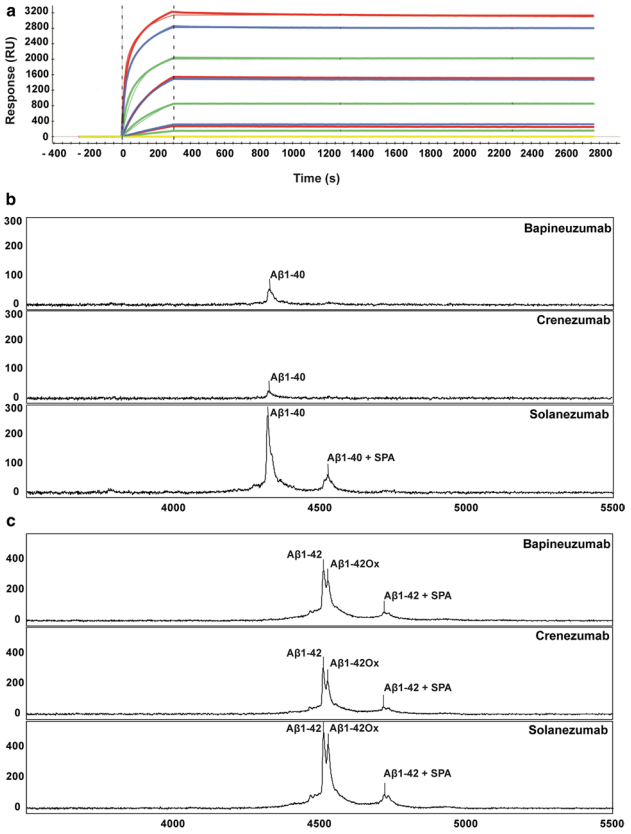


**Fig. 1** Recognition of synthetic Aβ species using bapineuzumab, crenezumab, and solanezumab. **a** Representative SPR binding response curves on the same coordinate axis for antibody binding to $Aβ_{1–40}$ captured on a NLH sensor chip at the C-terminus in PBS, pH 7.4, with 0.005 % (v/v) Tween-20. The maximum concentrations of bapineuzumab (*red*), solanezumab (*blue*), and crenezumab (*green*) are 2.9, 7.0, and 2.8 μM, respectively, with a series of tenfold dilutions. Only the three highest concentrations of each antibody and the blank are shown for clarity. *Thick lines* represent data points and the *thin lines* are fits obtained with the ProteOn Manager Software package. **b**, **c** Representative SELDI-TOF MS spectra of 50 pM synthetic $Aβ_{1–40}$ and $Aβ_{1–42}$ arising from analysis with bapineuzumab, crenezumab, and solanezumab

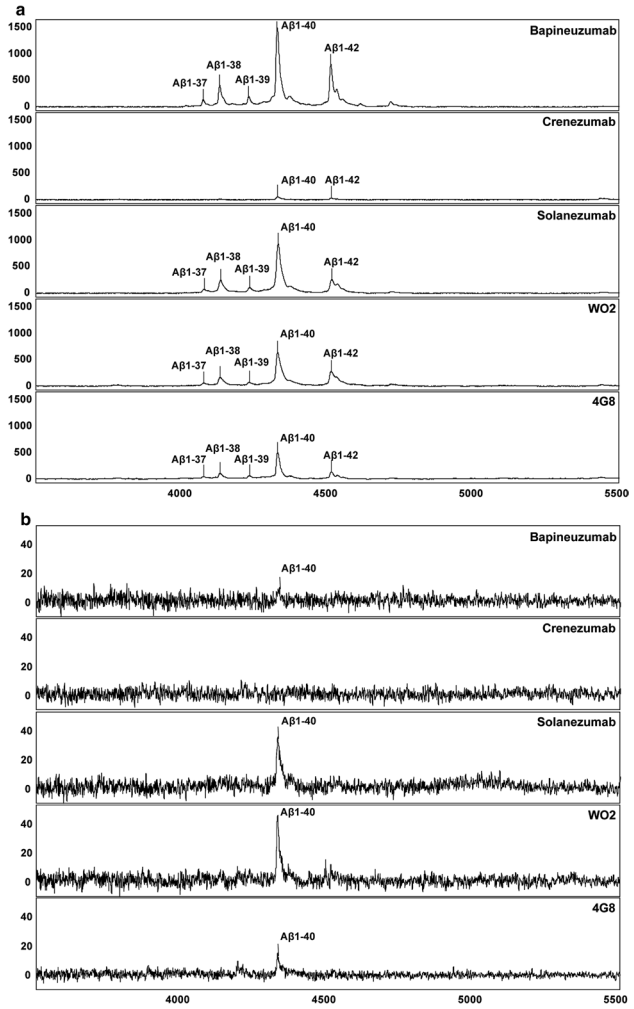


**Fig. 2** Spectral profiles of transgenic $APP_{SWE}$ mouse tissue. Representative SELDI-TOF MS spectra arising from the analysis of transgenic $APP_{SWE}$ (tg2576) mouse **a** brain homogenate ($n = 3$) and **b** plasma ($n = 3$) with bapineuzumab, crenezumab, solanezumab, and WO2

may have been due to the inhibition of aggregation or conformational change induced by the detergent. In either case, it highlights the pleomorphic nature of Aβ peptides and how care must be taken when interpreting affinity coefficients for these interactions.

With the antibodies conjugated to the sensor chip and Aβ peptides introduced as analytes, $k_d$ for the solanezumab and crenezumab was similar for all peptides tested, namely, $Aβ_{1–28}$, $Aβ_{1–40}$, $Aβ_{1–42}$, $Aβ_{3–42}$, $Aβ_{4–42}$, $pEAβ_{3–42}$, and $pEAβ_{11–42}$ which all contain the shared linear epitope. Bapineuzumab showed no change in $k_d$ for $Aβ_{1–28}$, $Aβ_{1–40}$, $Aβ_{1–42}$, but no binding was detected between the antibody and any Aβ species with an altered N-terminus (Supplementary Table 2). Bapineuzumab, solanezumab, and crenezumab when conjugated directly to the SELDI chip matrix readily detected synthetic $Aβ_{1–40}$ and $Aβ_{1–42}$ at 50 pM by SELDI-TOF MS (Fig. 1b, c).

## Antibodies bind Aβ in $APP_{SWE}$ transgenic mice

Samples collected from $APP_{SWE}$ transgenic mice, either cortical homogenate (n = 3) or plasma (n = 3), were incubated on SELDI-TOF arrays coupled with the therapeutic antibodies. Mass spectrometric analysis of these fractions showed that in $APP_{SWE}$ transgenic mice cortical samples bapineuzumab, solanezumab, WO2 and 4G8 all capture a near identical range of C-terminally truncated Aβ species: $Aβ_{1–37}$, $Aβ_{1–38}$, $Aβ_{1–39}$, $Aβ_{1–40}$, and $Aβ_{1–42}$ (Fig. 2a). Crenezumab only captured the dominant species $Aβ_{1–40}$ and $Aβ_{1–42}$ with lower peak intensities. With the exception of crenezumab, all the antibodies captured

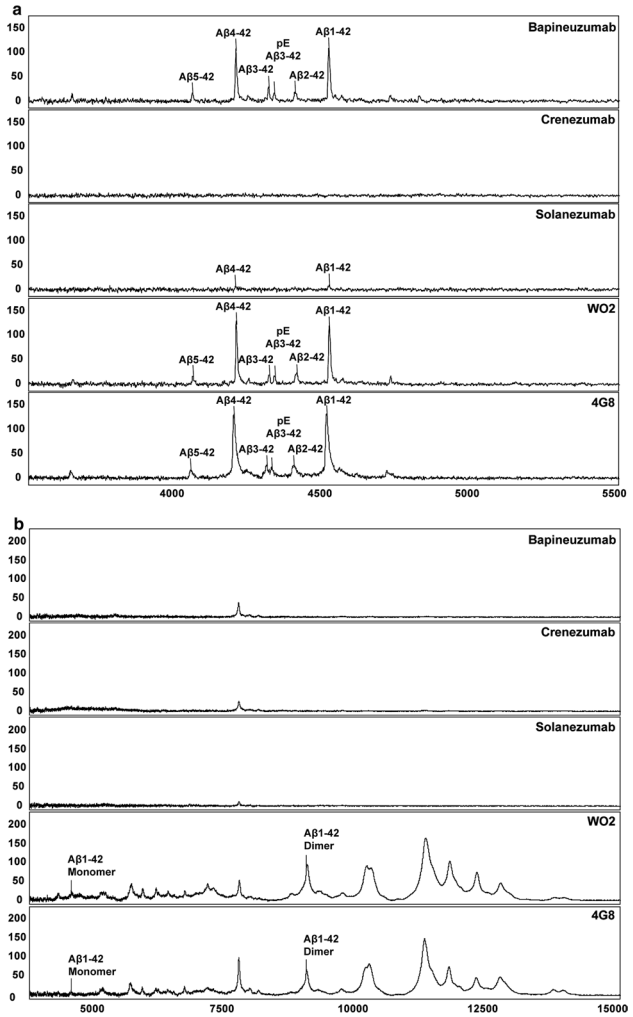


**Fig. 3** Spectra profiles of AD-affected human tissue Representative SELDI-TOF MS spectra arising from the analysis of AD-affected **a** brain homogenate ($n = 4$) and **b** the blood cellular fraction ($n = 4$) with bapineuzumab, crenezumab, solanezumab, WO2, and 4G8

$A\beta_{1–40}$ in plasma collected from $APP_{SWE}$ transgenic mice (Fig. 2b).

## Not all antibodies recognize Aβ in AD-affected human brain tissue

As with the samples collected from the transgenic animals, analysis of AD-affected cortical homogenate and plasma was conducted using SELDI-TOF MS. This experiment revealed significant differential activities between the antibodies when analyzing the AD-affected brain homogenates ($n = 4$; Fig. 3a). The resulting spectra indicated that bapineuzumab, WO2, and 4G8 captured a spectrum of N-terminally truncated Aβ peptides ranging from $A\beta_{5–42}$ through $A\beta_{1–42}$, with very strong signal-to-noise. In contrast, detection of Aβ species in the AD cortical homogenate when

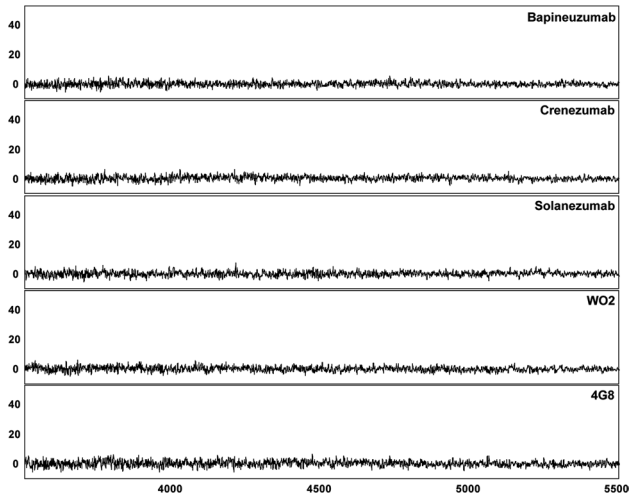


**Fig. 4** Spectral profiles of the AD-affected plasma. Representative SELDI-TOF MS spectra of plasma from AD patients demonstrated that all antibodies failed to resolve peaks consistent with known APP/Aβ species

using solanezumab and crenezumab was significantly lower with signals either being barely detectable above the noise or not detectable at all.

## Therapeutic antibodies do not recognize Aβ species in the blood cellular fraction

We have previously shown that the cellular fraction of blood is rich in Aβ-related biomarkers [22]. Here, analysis of the blood cellular fraction indicated that the monomeric and dimeric Aβ species observed using WO2 and 4G8 were not detected using bapineuzumab, crenezumab, and solanezumab ($n = 4$; Fig. 3b). A single peak around 8 kDa was captured using all three antibodies; however, this did not align with any reported fragments of APP or Aβ and was considered to be the result of non-specific interactions.

## There is no detectable Aβ in plasma

SELDI-TOF MS was also used for identification of Aβ species in AD-affected plasma samples ($n = 4$; Fig. 4). Bapineuzumab, crenezumab, solanezumab, and the laboratory standards WO2 and 4G8 were all unable to capture detectable levels of any Aβ species from AD-affected plasma samples, consistent with our earlier findings [11] and the short half-life of soluble Aβ in vivo [25]. The antibodies were all however, capable of binding synthetic $A\beta_{1–42}$ spiked into the AD-affected plasma at picomolar concentrations (Supplementary Fig. 1), indicating that the exchangeable pool of Aβ observed in the transgenic animal models is not reflected in sporadic AD.

**Table 1** Proteins identified following immunoprecipitation of AD plasma with crenezumab, and solanezumab

| Protein | Gene symbol | UniProt ID | $A\beta_{16–23}$ sequence alignment |
|---|---|---|---|
| | | | 16 **KLVFFAED** 23 |
| Probable ATP-dependent DNA helicase | HFM1 | A2PYH4 | 147 **KLV**N**FAED** 154 |
| Contactin-associated protein-like 3B | CNT3B | Q96NU0 | 240 **KLVFF**LNS 244 |
| 2-oxoglutarate and iron-dependent oxygenase domain-containing protein | OGFD1 | Q8N543 | 206 **KLVFF**EVS 210 |
| Cysteine-rich protein 3 | CRIP3 | Q6Q6R5 | 12 QP**VFFAE**K 16 |
| Solute carrier family 2, member 13 | SLC2A13 | Q96QE2 | 517 Y**LVFFA**PG 521[a] |
| Autophagy-related protein 9B | ATG9B | Q674R7 | 531 Q**LVFFA**GA 535 |
| Neurotrimin | NTM | Q9P121 | 279 **KL**I**FF**NVS 283[a] |
| Kelch domain containing 2 | KLHDC2 | Q9Y2U9 | 156 **KL**I**FF**GGY 160[a] |
| Interleukin-12 receptor beta-1 | IL12RB1 | P42701 | 547 W**L**I**FFA**SL 551[a] |
| Peroxisomal bifunctional enzyme | EHHADH | Q08426 | 272 QYA**FFAE**R 275[a] |
| Cardiomyopathy associated 3 | XIRP2 | A4UGR9 | 24 PESD**FAED** 27[a] |
| Zinc finger protein 429 | ZN429 | Q86V71 | 81 CSH**FAED** 84 |

Sequence homology is highlighted by the bold and underlined text

[a] Mapped in Plasma Proteomics Database

### Solanezumab and crenezumab bind non-Aβ related species in plasma

Given the poor performance of the therapeutic antibodies in capturing Aβ in blood, we used immunoprecipitation (IP) in conjunction with LC-MS/MS to better characterize the mAb binding profiles in human plasma. No Aβ peptides were detected with any of the antibodies. However, bapineuzumab was the only antibody to pull down a construct from the C-terminal cytoplasmic tail of APP (Supplementary Table 3). Given the N-terminal and conformational requirements of bapineuzumab for Aβ, this species was likely the BACE-derived Aβ precursor C99. Using crenezumab and solanezumab, the IPs pulled down and identified just over 200 proteins in AD-affected plasma not seen by bapineuzumab or beads alone. Twelve of these exhibited sequence identity with the linear epitope (KLVFFAED) central to the putative Aβ epitopes recognized by crenezumab and solanezumab (Table 1).

## Discussion

One of the key driving forces for the amyloid cascade hypothesis is that the accumulation of cortical Aβ precedes the clinical onset of AD by upwards of 20 years [26, 27]. Clinical validation of this hypothesis requires that lowering cortical Aβ burden should lead to cognitive improvements or that the reduction of pre-clinical Aβ accumulation should prevent or delay the onset of disease. However, to date anti-Aβ therapies have failed in the clinical setting, raising questions about the validity of the amyloid hypothesis of AD and lending strength to the argument that therapeutic interventions in the mild to moderate stages of AD are simply a case of too little, too late [28–30]. It could be argued, however, that the amyloid hypothesis has not yet been adequately tested, as the therapeutic compounds thus far selected for clinical evaluation have suffered from a number of fundamental flaws, including an inability to cross the blood–brain barrier, toxic side effects, questionable mechanisms of action [30], and now questions regarding whether appropriate target engagement is occurring.

Given this history, the field has now determined that the best time to target Aβ accumulation is prior to symptom onset before significant and irreversible damage has been done to the brain. For this strategy to work, the chosen drug(s) must be capable of reducing the pool of Aβ that is thought to drive the disease, i.e., brain-derived Aβ. Studies in transgenic animals have shown that bapineuzumab, crenezumab, and solanezumab engage the target in these models successfully reducing Aβ burden [8, 10, 12]. Consistent with these data utilizing SELDI-TOF MS technology, our analysis showed that all the antibodies could capture Aβ from transgenic mouse brain tissue (Fig. 2a). When we examined human AD brain tissue, bapineuzumab was able to capture an array of Aβ species, but at the same time both solanezumab and crenezumab were very poor at capturing the Aβ species present in the brain tissue (Fig. 3a), indicating a different spectrum of activity for solanezumab and crenezumab between mouse and human tissue. While these results were surprising, they were in line with reported clinical outcomes in that bapineuzumab can successfully reduce Aβ burden as detected by positron emission tomography imaging studies [5], while solanezumab does not [31].

Based on mouse studies, it has been suggested that solanezumab does not work directly on brain Aβ, but

instead works as a peripheral sink targeting peripheral Aβ which in turn lowers CNS Aβ by mass action. Indeed when we examined plasma from tg2576 mice, solanezumab was able to detect $Aβ_{1–40}$ in plasma (Fig. 2b). However, none of the therapeutic antibodies were able to detect Aβ in either the plasma or the cellular fraction from human AD subjects (Figs. 3b, 4). The first thing to note from these data is that Aβ profiles in the mouse blood are completely different to those of human blood, once again highlighting that caution must be exercised when extrapolating results from transgenic animals into humans. Furthermore, it raises the question; why did these antibodies fail to capture any Aβ species? Our SPR data confirmed that all the antibodies have a high affinity for Aβ (Fig. 1) and the ability to capture Aβ species in complex biological milieu was confirmed by our examination of the mouse tissue (Fig. 2). So if affinity is not the issue, perhaps it is a question of the antibodies' specificity for Aβ?

All of the therapeutic antibodies were raised against linear epitopes within the Aβ sequence. It is likely that these epitopes are at least partially present in other proteins and that the resulting cross reactivity with these proteins diminishes the ability of the antibodies to target Aβ. Immunoprecipitation experiments from human plasma (Table 1) indicated that cross reactivity does indeed occur and is likely to explain the poor ability of solanezumab and crenezumab to capture Aβ species in human tissue. The similar cross reactivity profiles of solanezumab and crenezumab were consistent with their sequence similarities. One of the proteins pulled down by both solanezumab and crenezumab was the IL12 receptor; this is interesting as a recent publication showed that modulation of the IL12 signaling pathway resulted in cognitive improvements in a transgenic mouse model of AD [32]. It should be noted that a driving force for the selection of solanezumab as a drug candidate for the preventative trials was the post hoc analysis that showed a small, but significant improvement in cognition in the mild AD subjects in Phase 3 trials.

In summary, two of the leading drug candidates for preventative clinical trials are effectively the same antibody indicating a lack of diversity in therapeutic strategies. These antibodies poorly recognize Aβ in human tissue as a result of cross reactivity, which is a well-known problem for therapeutic antibodies raised against linear target sequences [33–35]. This lack of specificity and target engagement for Aβ does not bode well for the prospects of success of these drugs in the preventative trials that are ultimately likely to validate the amyloid cascade hypothesis for AD. Bapineuzumab while raised against a linear epitope of Aβ, requires a specific conformation for binding [3]; this gives the antibody a degree of specificity and as a result this drug has clinically demonstrated target engagement, but unfortunately toxicity issues prevent further evaluation of this drug. Over stimulation of microglia or removal of Aβ embedded in the vascular architecture of the AD brain has been suggested to explain bapineuzumab toxicity. This would recommend the use of an Fc-modified form of bapineuzumab, AAB-003 recently developed by Pfizer. Ultimately, if the amyloid cascade hypothesis is to be validated in a pre-symptomatic cohort, the drug utilized must be clinically proven to safely reduce cortical Aβ burden.

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