Relationship between therapeutic activity and preferential targeting of toxic soluble aggregates by amyloid-beta-directed antibodies

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ABSTRACT

Amyloid-beta (Aß)-directed antibodies tested clinically for therapeutic activity against Alzheimer's disease (AD) have shown varying degrees of efficacy. Although all of these antibodies target the Aß peptide, their binding profile to different molecular species of Aß (monomers, oligomers, fibrils) differs and may underly the observed variability in clinical outcomes. Surface plasmon resonance (SPR) was used to conduct a side-by-side comparison of the binding of various Aß-directed antibodies to monomers and soluble low and high molecular weight Aß oligomers from AD brains. Immunohistochemistry was performed to assess reactivity with Aß fibrils in plaque. Non-selective, pan-Aß reactive antibodies such as crenezumab and gantenerumab, which have failed to produce a clinical benefit, bound all forms of Aß tested. In a competition assay aimed at replicating the *in vivo* abundance of monomers in the blood and the central nervous system (CNS), these antibodies lost the ability to bind toxic AD brain oligomers when exposed to even low concentrations of monomers. In contrast, aggregate-selective antibodies such as aducanumab, lecanemab and donanemab, which have reported a clinical benefit, showed reduced monomer binding and a greater ability to withstand monomer competition suggesting a relationship between therapeutic activity and preferential targeting of toxic soluble aggregates. Of the antibodies in earlier stages of clinical testing, ACU193 and PMN310 displayed the greatest ability to retain binding to toxic AD brain oligomers when faced with high monomer concentrations while PRX h2731 was highly susceptible to monomer competition. Plaque binding was observed with all aggregate-reactive antibodies with the exception of PMN310 which was strictly selective for soluble oligomers. While a correlation has been observed between antibody plaque binding/clearance and reported increases in the risk of ARIA, plaque binding did not translate into clinical benefit in the case of gantenerumab. Overall, these results suggest that selectivity for soluble toxic Aß oligomers may be a driver of clinical efficacy, with a potential reduced risk of ARIA if engagement with plaque is minimized.

INTRODUCTION

Strong genetic and experimental evidence supports a causative role for amyloid-beta (Aß) in the pathogenesis of Alzheimer's disease (AD) in agreement with the demonstrated ability of Aß-directed antibodies such as aducanumab, lecanemab and donanemab to slow cognitive decline in pivotal clinical trials (1-6). What remains unclear is the exact nature of the Aß species being targeted by various antibodies and their relative contribution to clinical efficacy. While there exists a seeming correlation between cognitive benefit and antibody-mediated plaque clearance as measured by PET imaging, the same antibodies can also mediate clearance of soluble toxic Aß species such as oligomers. Because there are currently no available standardized methods for clinical measurement of these toxic Aβ species, it has been difficult to evaluate potential correlations with the efficacy of treatment.

A large body of experimental and clinical evidence suggests that soluble toxic Aß aggregates, rather than insoluble fibrils and plaque, are the primary drivers of synaptic dysfunction, neuronal loss and cognitive decline in AD patients (7-12). For example, genetic data indicate that the APP Osaka (E693Δ), and APP p.D678N mutations cause rapidly progressive dementia with abundant soluble toxic Aβ species, and little or no Aβ plaque as measured by PiB PET (13, 14). As such, the intended mechanism of action for antibodies such as lecanemab, ACU193 and PMN310 is targeting these toxic soluble species. Since soluble aggregates of Aß are heterogeneous, questions also arise as to which subset(s) of aggregates are being targeted and how these antibodies might be responsible for efficacy. In this study, a side-by-side comparison of the binding profile of various Aß-targeted antibodies to monomers, low and high molecular weight AD brain oligomers, and plaque was conducted and compared with known clinical outcomes. The results suggest that clinical efficacy may be related to both the ability of Aß-directed antibodies to bind soluble toxic Aß aggregates and to a degree of specificity allowing for binding soluble toxic Aß aggregates in the presence of Aß monomers.

RESULTS

Aß-directed antibodies bind to soluble species in AD brain extract

The neurotoxicity of soluble Aß aggregates of various sizes ranging from dimers to protofibrils has been described *in vitro* and *in vivo* by multiple investigators (9, 15-17). Preferential targeting of these soluble species, as opposed to insoluble fibrils, has been proposed as the primary mechanism underlying lecanemab's clinical efficacy (6, 18). We therefore compared the binding of lecanemab and other clinical-stage antibodies to soluble species of Aß derived from AD brains using surface plasmon resonance (SPR). Equivalent amounts of antibodies were immobilized on a sensor chip and soluble AD brain extract fractionated by size-exclusion chromatography into a low molecular (LMW) fraction excluding monomers (~8-70 kDa, oligomer-enriched) and a high molecular weight (HMW) fraction (~140-700 kDa, protofibril-enriched) was injected over the surface (Fig. 1). As shown in figure 2, lecanemab displayed binding to both soluble AD brain aggregate fractions in agreement with previous reports showing strong binding of lecanemab to oligomers (defined by the authors as <75 kDa), small protofibrils (defined as 75-400 kDa) and large protofibrils (defined as 300-5,000 kDa) (19, 20). The same reports indicated that the Aß species with the most toxicity resided in the 20-80 kDa and 80-500 kDa fractions, overlapping with the LMW and HMW fractions tested herein thereby suggesting that reactivity against both fractions may contribute to the protective activity of lecanemab. While emphasis has been placed on the reactivity of lecanemab with protofibrils over 75 kDa, the binding profile of this antibody to Aß species in AD brain appears broader and it likely targets a range of toxic soluble species of various sizes as opposed to a specific subpopulation with a defined molecular weight cut-off.

Other antibodies (crenezumab, PRX h2731, donanemab, aducanumab and ACU193) showed comparable levels of binding to soluble species in both the LMW and HMW fractions of AD brain extract (Fig. 2). PMN310, like lecanemab, showed binding to both fractions with a trend for preferential reactivity with the LMW toxic oligomer-enriched fraction.

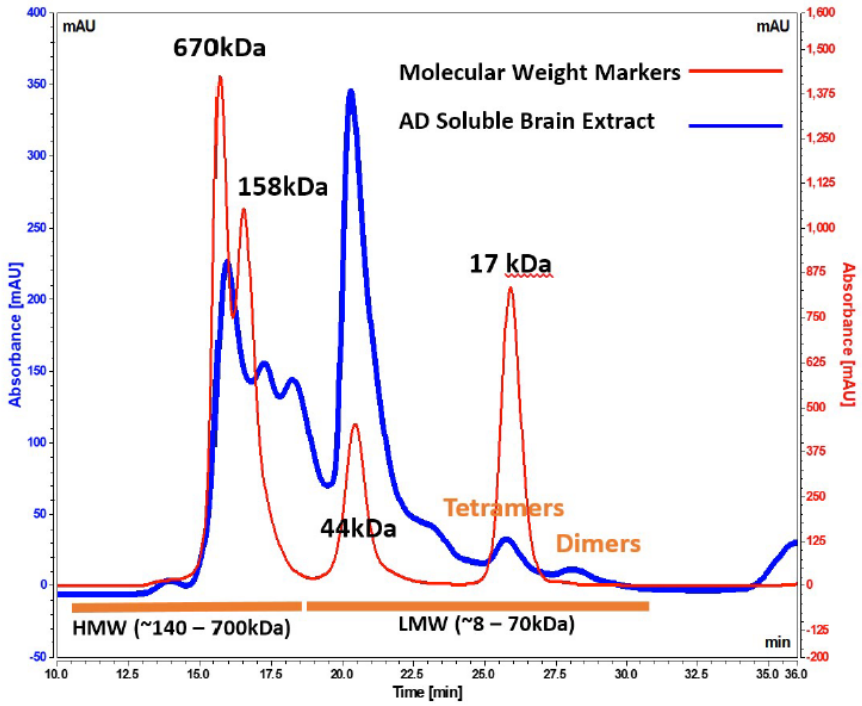

Figure 1. Size-exclusion chromatogram of soluble AD brain extract. Fractionated soluble brain extract was pooled into a low molecular weight (LMW) fraction of ~8-70 kDa and a high molecular weight (HMW) fraction of ~140-700 kDa. Aβ monomers (4.5 kDa) were excluded.

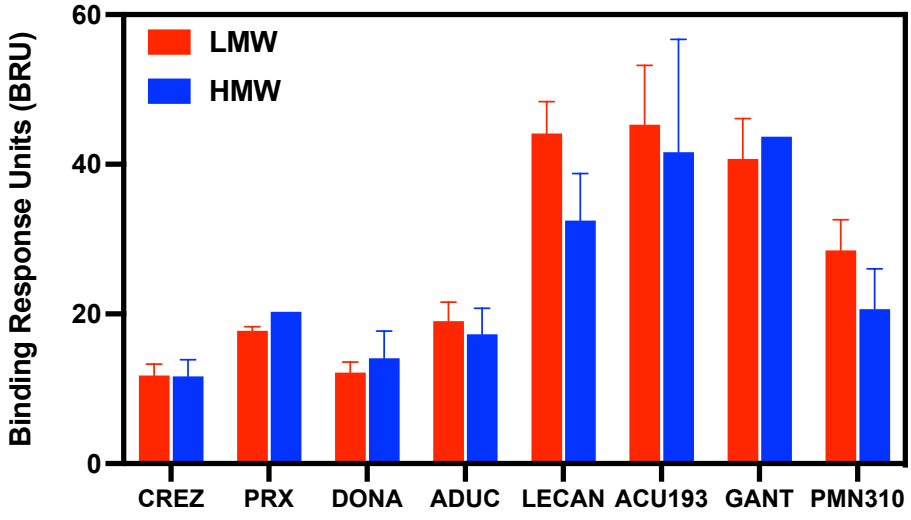

Figure 2 – Binding of Aß-directed antibodies to soluble species in AD brain extract. SPR measurement of immobilized antibody binding to the LMW and HMW fractions of soluble AD brain extract. Results shown are the mean ± SEM of combined data from 10 separate studies for the LMW fraction and 7 studies for the HMW fraction. Not all antibodies were tested in all

studies. CREZ: crenezumab (LMW - 8 studies, HMW - 5 studies), PRX: PRX h2731 (LMW - 2 studies, HMW - 1 study), DONA: donanemab (LWW - 10 studies, HMW - 7 studies), ADUC: aducanumab (LMW - 10 studies, HMW - 7 studies), LECAN: lecanemab (LMW - 10 studies, HMW - 6 studies), ACU193 (LMW - 9 studies, HMW - 6 studies), GANT: gantenerumab (LMW - 5 studies, HMW - 1 study, PMN310 (LMW - 10 studies, HMW - 7 studies).

Ability to avoid monomer competition corresponds with clinical efficacy

Clinical results obtained to date indicate that therapeutic efficacy is significantly affected by the binding profile of Aß-directed antibodies (21). Antibodies with pan-reactivity such as gantenerumab and crenezumab which bind all species of Aß, including toxic oligomers, did not achieve clinical benefit. These results suggest that efficacy may depend on antibody selectivity and the ability to avoid interaction with abundant, non-toxic monomers that can reduce the effective dose available for targeting pathogenic soluble species which are present at concentrations at least 1,000 fold lower in the brain (16, 22-24). This concept was explored in SPR binding studies to determine whether a relationship exists between positive clinical data and the ability of an antibody to retain binding to soluble oligomers from AD brain extract in the face of monomer competition.

First, the binding of Aß-directed antibodies to increasing concentrations of Aß monomers (0.08-5.0 μM) was measured by SPR (Fig. 3). Pan-Aß reactive antibodies such as crenezumab, gantenerumab and PRX h2731 showed high levels of binding, even at the lowest concentration of monomers tested. Antibodies with more selectivity for aggregated forms of Aß such as donanemab, aducanumab and lecanemab showed markedly lower levels of monomer binding, although lecanemab still displayed relatively robust binding at the highest concentrations of monomers. PMN310 and ACU193, both designed to selectively target oligomers, showed low monomer binding across the range of concentrations.

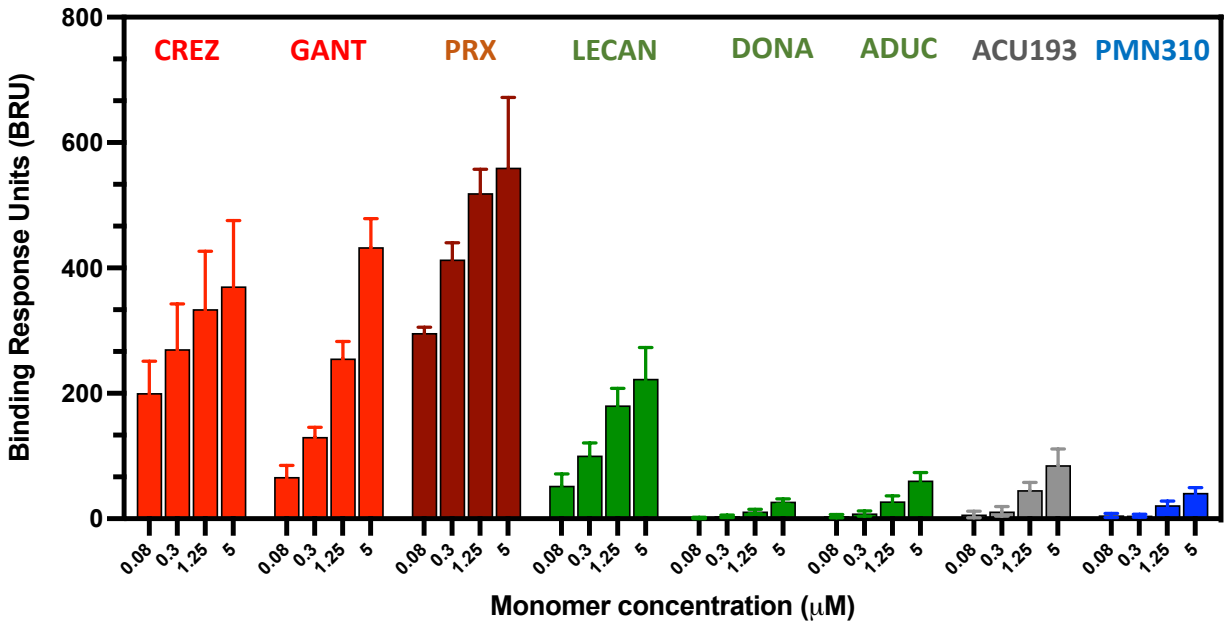

Figure 3. Binding of Aß-directed antibodies to monomers. SPR measurement of immobilized antibody binding to Aß monomers. Results shown are the mean ± SEM of combined data from 6 separate studies. Not all antibodies were tested in all studies. CREZ and GANT - 4 studies, PRX - 2 studies, LECAN, DONA , ADUC, ACU193 and PMN310 - 6 studies.

Binding of antibodies to the LMW and HMW fractions from soluble AD brain extract was then measured either in the absence of Aß monomers (100% binding response) or in the presence of increasing concentrations of monomers to mimic the competition that antibodies will encounter in the circulation and in the brain after intravenous administration. Binding to the LMW fraction of soluble AD brain extract is of particular interest since it has been reported to be enriched for toxic oligomers as assessed by inhibition of long-term potentiation (11), induction of cognitive deficit in rats (8, 25), and activation of microglia (26). In side-by-side comparison studies, binding of oligomers by antibodies with pan-Aß reactivity such as crenezumab, gantenerumab and PRX h2731 was strongly inhibited by low concentrations of monomers thereby reducing the amount of antibody available to bind oligomers in the LMW (Fig. 4a) or HMW (Fig. 4b) fractions of soluble AD brain extract. In contrast, antibodies with greater selectivity for aggregated Aß were better able to withstand monomer competition and retained measurable binding to LMW and HMW oligomers even at high concentrations of monomers. Notably, PMN310 and ACU193 retained the highest level of oligomer binding.

Based on the clinical experience to date, these results suggest a relationship between clinical benefit and the ability of Aß-directed antibodies to avoid diversion by monomers and target toxic oligomers. The pan-reactive Aß antibodies crenezumab and gantenerumab, which were highly susceptible to monomer competition, have failed in pivotal clinical trials (27, 28) while antibodies such as aducanumab, lecanemab and donanemab, which retained binding to oligomers in the face of monomer competition, provided a clinical benefit (2, 5, 6).

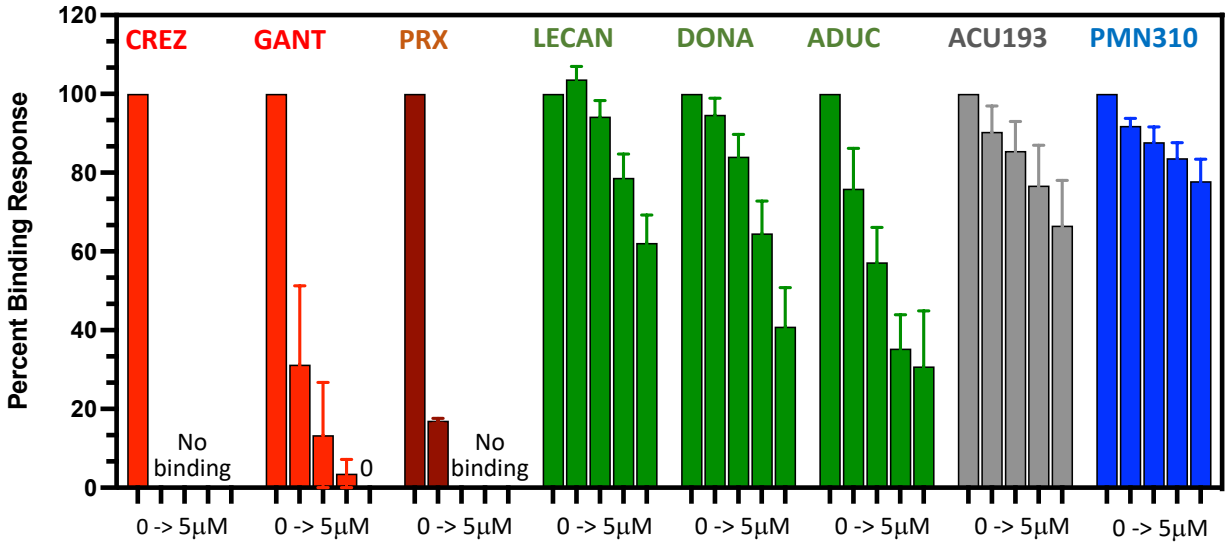

Figure 4a. Binding to LMW brain oligomers in the face of monomer competition. SPR measurement of immobilized antibody binding to the LMW fraction of soluble AD brain extract after pre-exposure to monomer concentration ranging from 0 (100% binding), 0.08, 0.3, 1.25, and 5 µM. Percent binding response was calculated as: [(BRU) with monomers) / (BRU without monomers)] X100. Results shown are mean ± SEM of combined data from 6 separate experiments. Not all antibodies were tested in all studies. CREZ and GANT - 4 studies, PRX - 2 studies, LECAN, DONA , ADUC, ACU193 and PMN310 - 6 studies.

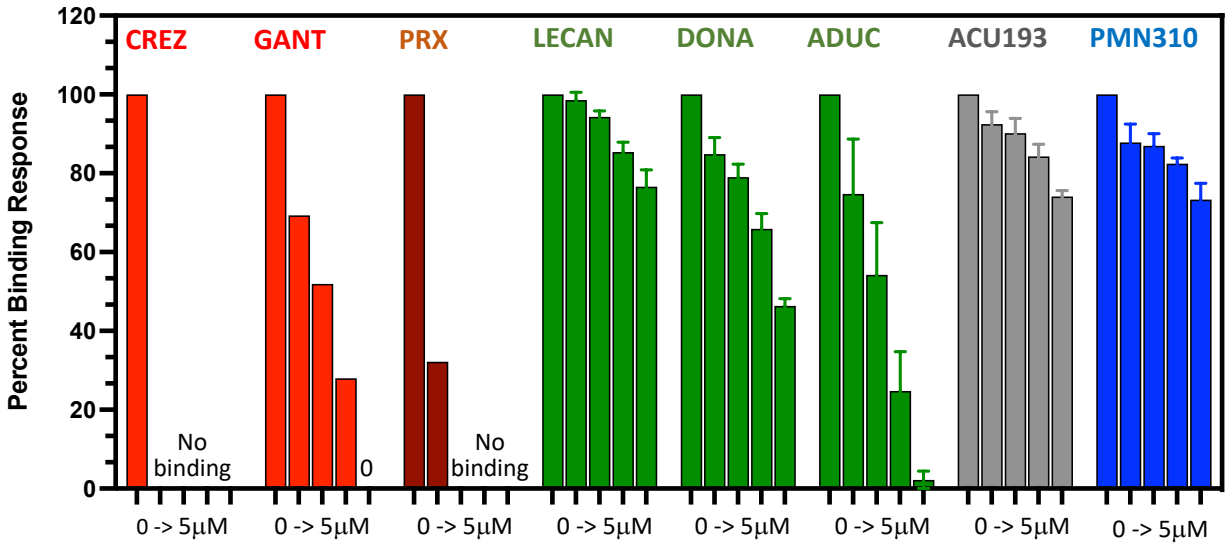

Figure 4b. Binding to HMW brain oligomers in the face of monomer competition. SPR measurement of immobilized antibody binding to the HMW fraction of soluble AD brain extract after pre-exposure to monomer concentrations ranging from 0 (100% binding), 0.08, 0.3, 1.25, and 5 µM. Percent binding response was calculated as: [(BRU) with monomers) / (BRU without monomers)] X100. Results shown as mean ± SEM of combined data from 3 separate experiments. LECAN, DONA, ADUC, ACU193 and PMN310 were tested in all 3 studies, CREZ, GANT and PRX in 1 study.

Binding of Aß-directed antibodies to plaque

The reactivity of Aß-directed antibodies with insoluble fibrillar deposits in AD brain sections was assessed by immunohistochemistry (IHC). Most antibodies (gantenerumab, aducanumab, lecanemab, donanemab, PRX h2731 and ACU193) showed staining of parenchymal plaque and vascular deposits of Aß (Fig. 6). Where clinical data are available, binding to insoluble Aß, in particular vascular deposits, has been associated with an increased incidence of amyloid-related imaging abnormalities (ARIA) ranging from ~15-35% depending on the antibody (Fig. 6) (2, 28-32). The underlying mechanism is believed to involve local inflammation triggered by the interaction of bound antibodies with Fc receptors on microglial cells leading to their activation. The consequent release of pro-inflammatory mediators, together with potential weakening of the vascular wall following amyloid removal, is thought to result in brain edema (ARIA-E) and microhemorrhages (ARIA-H) (33-35). In agreement with this premise, solanezumab which targets monomers and does not bind plaque (Fig.6), did not produce an increased incidence of ARIA (36) while AL002, a TREM2 agonist antibody that directly activates microglial cells, was

associated with ARIA in a recent Phase 2 clinical trial (~27% ARIA-E, 29% ARIA-H) (37). Notably, PMN310 which is selective for soluble toxic Aß oligomers did not produce any detectable staining of plaque or vascular deposits in AD brain sections (Fig. 6). The ability of PMN310 to avoid both monomer and plaque binding would be expected to increase the available effective dose against toxic oligomers. Importantly, it may also reduce the risk of ARIA-E and ARIA-H observed with other Aß-directed antibodies. This premise is supported by preclinical toxicology studies in which weekly dosing of a murine version of PMN310 in transgenic *B6.Cg-App*$^{tm1.1Dnli}$*/J* mice at 800 mg/kg for 26 weeks did not cause brain hemorrhages upon microscopic examination using Perl's Prussian Blue staining to detect the presence of hemosiderin (Fig. 7).

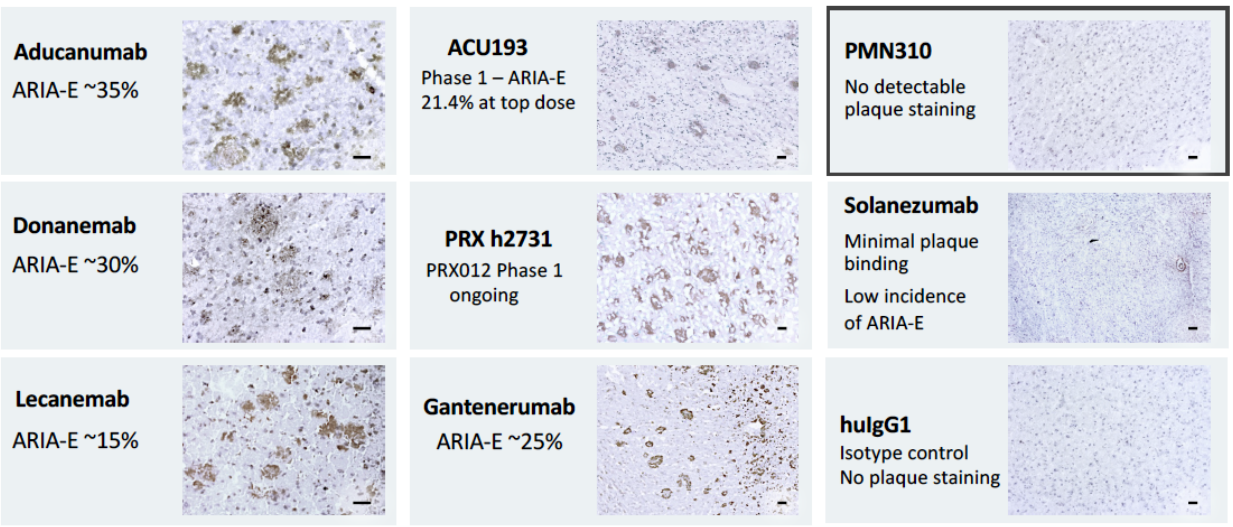

Figure 6. Reactivity of Aß-directed antibodies with insoluble fibrils. Representative images of plaque staining in brain sections from AD frontal cortex. All antibodies tested at the same concentration of 1 μg/ml. Scale bars = 50 μM.

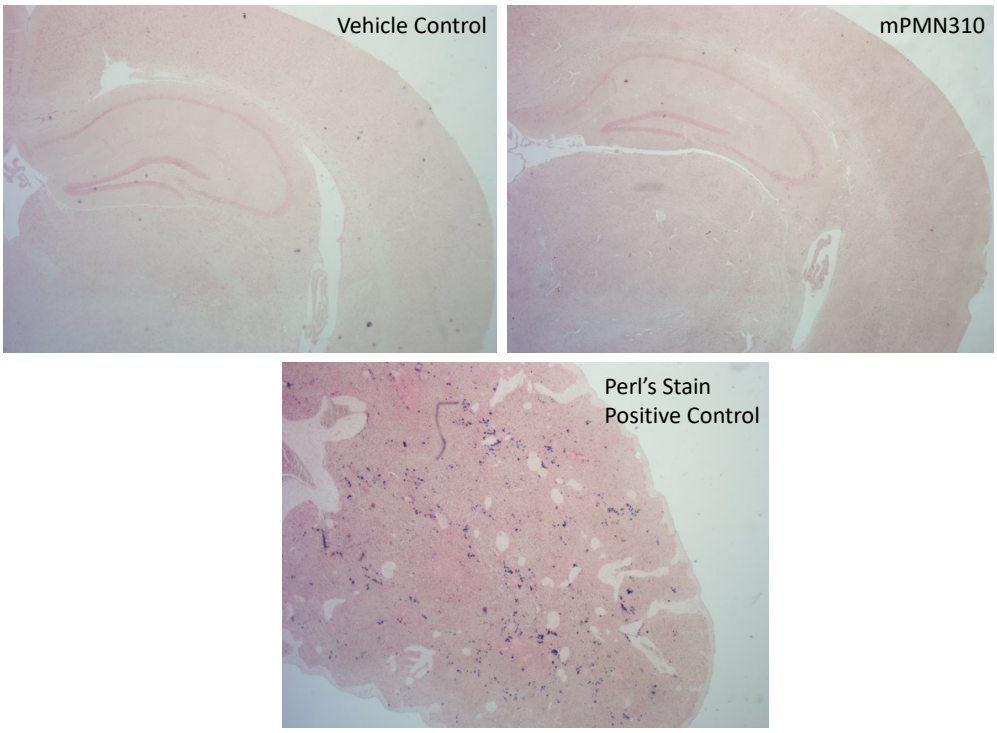

Figure 7. Treatment of *B6.Cg-App$^{tm1.1Dnli}$/J* mice at 800 mg/kg/week for 26 weeks does not cause brain hemorrhages. Sections of mouse brains were cut at a nominal thickness of 4 µm to 5 µm and stained with Perl's Prussian Blue stain to detect the presence of hemosiderin as an indicator of hemorrhage in the brain. An unrelated spleen sample was used as a positive control to validate the staining procedure. Brain sections from 29 vehicle control mice and 29 murine PMN310 (mPMN310)-treated mice were examined (14 males and 15 females in each group). Representative images at 25X magnification are shown.

DISCUSSION

As described here, toxic soluble Aß aggregates likely exist as a continuum of oligomers and protofibrils of various sizes as opposed to discrete populations with defined molecular weights. Based on the observed relationship between clinical efficacy and the ability to bind oligomers in the face of monomer competition, we propose that the efficacy of Aß-directed antibodies may depend on achieving an effective dose capable of clearing soluble toxic species spanning a range of molecular weights. Plaque clearance may also be beneficial but is associated with an increased risk of ARIA. Importantly, clinical data suggest that removing plaque is not sufficient to inhibit disease progression. For example, gantenerumab failed to achieve a clinical benefit despite its ability to clear plaque (27). Similarly, treatment gap data collected between trial completion and initiation of an open label extension (OLE) with lecanemab and aducanumab

have shown worsening of cognition after cessation of treatment even though plaque burden had been reduced and remained flat during the gap period (38, 39). These results suggest that removal of soluble toxic oligomers may be the primary driver of clinical benefit and must be sustained for continued efficacy.

Phase 1 clinical testing of ACU193, an antibody reported to have approximately 500-fold greater selectivity for Aβ oligomers over monomers and more than 85-fold greater selectivity for oligomers vs fibrillar Aβ, showed amelioration of biomarkers associated with AD pathology (pTau181, neurogranin, Aβ42/40 ratio, VAMP2, GFAP) after 3 months of dosing in patients with early AD (32). Plaque reduction was observed in the high dose cohorts (60 mg/kg Q4W and 25 mg/kg Q2W) possibly due to direct binding of ACU193 to plaque or potentially involving other mechanisms such as a shift in the oligomer-fibril equilibrium or a change in the milieu favoring microglial removal of plaque upon neutralization of inflammatory oligomers. Treatment with ACU193 was associated with a 21.4% incidence of ARIA-E at the highest dose of 60 mg/kg (combined single ascending dose and multiple ascending dose groups), a much higher dose than that used with other plaque-binding antibodies. The use of a human IgG2 isotype with reduced effector function *vs* the human IgG1 effector isotype of other antibodies may potentially have played a role in dampening inflammatory responses that can trigger ARIA following binding to Aβ plaque/vascular deposits.

Of all the antibodies tested in this study, only PMN310 selectively targeted oligomers without cross-reacting with monomers or plaque (Figs. 2, 3, 6, Table 1). Clinical Phase 1a testing of PMN310 in normal healthy volunteers was initiated in November 2023 (NCT06105528) and may provide the basis for a true test of the "oligomer hypothesis" without confounding cross-reactivity with other Aß species.

Antibody	Binding to oligomers vs monomer competition	Binding to plaque	Plaque clearance	ARIA-E	Clinical benefit in pivotal trials
Solanezumab	-	-	-	-	-
Crenezumab	-	+/-	-	+/-	-
Gantenerumab	-	+	+	+	-
Aducanumab	+	+	+	+	+
Lecanemab	+	+	+	+	+
Donanemab	+	+	+	+	+
ACU193	++	+	+	+	TBD
PRX h2731	-	+	TBD	TBD	TBD
PMN310	++	-	TBD	TBD	TBD

Table 1 – Summary of findings and clinical experience

MATERIALS & METHODS

Reagents

The monoclonal antibodies solanezumab, crenezumab, gantenerumab, aducanumab, lecanemab, donanemab, ACU193 and PRX h2731 were produced by Creative Biolabs (Upton, NY, USA) based on published sequences. PMN310, a humanized IgG1 monoclonal antibody selective for toxic Aβ oligomers (40) was manufactured by KBI Biopharma (Durham, NC, USA). The mouse IgG2a version of PMN310 was produced by Wuxi Biologics (Hong Kong, China). Hexafluoroisopropanol (HFIP)-treated recombinant Aβ42 and Aβ40 peptides (rPeptide, Watkinsville, GA, USA) was reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Canada, Oakville, ON) to give a stock concentration of 5 mM. Oligomers of Aβ42 were prepared by diluting the peptide solution in phenol red-free F12 medium (Thermo Fisher, Waltham MA, USA) to a final concentration of 100 µM and incubated for 24 h at 4°C followed by immediate use or storage at -80°C. Aβ40 which is less prone to aggregation, was used in

monomer competition assays. Human IgG1 isotype control was purchased from BioLegend (San Diego, CA, USA).

Brain extracts

Brain tissues from 12 different human AD patients were provided by Dr. Ian Mackenzie at the University of British Columbia, Vancouver, Canada. Informed consent for tissue collection at autopsy and neurodegenerative research use was obtained from the legal representative in accordance with local institutional review boards. The clinical diagnosis of AD was based on NINCDS-ADRDA criteria. Samples from frontal cortex were weighed and submersed in ice-cold Tris-buffered saline (TBS) (20% w/v) with EDTA-free protease inhibitor cocktail (Roche Diagnostics, Laval QC, Canada), and homogenized using an Omni tissue homogenizer (Omni International Inc, Keenesaw GA, USA), 3 x 30 sec pulses with 30 sec pauses in between, all performed on ice. Homogenates were then subjected to ultracentrifugation at 100,000xg for 60 min. Supernatants (soluble extracts) were collected, aliquoted and stored at -80ºC. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay. Pools of brain extracts from 3-5 patients were used in each analysis.

Size exclusion chromatography

Pooled soluble brain extracts were injected at 0.5 ml/min through a Superdex 75 (10/300) HPLC column (GE Healthcare Life Sciences, Pittsburg PA, USA) for 50 min and 0.25 ml fractions were collected. Molecular weight (MW) markers (Bio-Rad Laboratories, Mississauga ON, Canada) were run separately. Protein peaks were monitored by absorbance at O.D. 280 nm. Fractions corresponding to a MW of ~8kDa to ~70kDa were pooled into a low molecular weight (LMW) fraction. Aβ monomers (MW ~4.5kDa) were excluded from the LMW fraction. Fractions corresponding to a MW of >140kDa to ~700kDa were pooled into a high molecular weight (HMW) fraction. The LMW and HMW fractions were concentrated and total protein concentration was determined in a BCA assay. The fractions were then diluted to 100 µg/ml in phosphate-buffered saline, 3 mM EDTA, 0.05% surfactant P20 (PBS-EP) (GE Healthcare Life Sciences, Pittsburg PA, USA) containing BSA (2 mg/ml) for surface plasmon resonance (SPR) analysis.

Surface plasmon resonance analysis

Surface plasmon resonance measurements were performed using a Molecular Affinity Screening System (MASS-2) instrument (Bruker Daltronics, Billerica, MA, USA). Antibodies were immobilized on high amine capacity sensorchips at a density of approximately 9,000-11,000 response units (RUs). To assess the binding of antibodies to native AβO, LMW and HMW SEC fractions of pooled soluble human AD brain extracts (100 μg/ml) were injected over the immobilized antibodies at 10 μl/min for 8 minutes. The binding responses from the resultant sensorgrams were double-referenced against unmodified reference surfaces and blank buffer injections. Binding response units (BRUs) were collected 30 seconds post-injection stop during the dissociation period. In monomer competition studies, Aβ40 monomers (0.08 μM - 5 μM) were pre-injected over immobilized antibodies at 12.5 μl/min for 15 minutes followed by injection of brain extract (100 μg/ml) at 12.5 μl/min for 10 minutes. Percent binding response was calculated as: [(BRU) with monomers) / (BRU without monomers)] X100.

Immunohistochemistry

Fresh-frozen, frontal cortex AD brain sections (25 μm-thick) were mounted onto charged glasses slides and fixed by immersion in -20 °C acetone for 20 min. Between subsequent steps, slides were washed extensively in Tris-buffered saline (TBS, pH 7.4) containing 0.2% TritonX-100. To suppress endogenous peroxidase activity, sections were treated with 0.3% H_20_2, followed by incubation in TBS buffer containing 10% normal goat serum (NGS), 3% BSA, and 0.2% Triton-X-100 to block non-specific binding sites. Next, an avidin/biotin blocking kit (Vector Laboratories, Burlington, ON, Canada) was employed as per the manufacturer's instructions. Endogenous immunoglobulins were blocked by incubating tissue in Fab fragment goat anti-human IgG (H+L) (Jackson ImmunoResearch Labs, West Grove, PA, USA; 100 μg/ml) for 2 h at room temperature. Primary antibodies (solanezumab, gantenerumab, aducanumab, lecanemab, donanemab, ACU193, PRX h2731 and PMN310), were adjusted to 1 μg/ml in background-reducing antibody diluent (Agilent, Santa Clara, CA, USA) and applied overnight at 4 °C in a humidified chamber. Biotin-conjugated goat anti-human IgG (Fab')2 antibody (Abcam, Waltham, MA, USA; 1 μg/ml) was subsequently added for 1 h at room temperature. Staining was visualized using the ABC method, with a Vectastain kit (Vector Laboratories) and diaminobenzidine (DAB) as chromogen. Nuclei were counterstained using methyl green or

hematoxylin QS (Vector Laboratories) and sections were dehydrated, cleared, and coverslipped. The slides were examined under a light microscope (Zeiss Axiovert 200M, Carl Zeiss Toronto ON, Canada) and representative images were captured using a Leica DC300 digital camera and software (Leica Microsystems Canada Inc., Vaughan ON, Canada).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.

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Author contributions

NRC, EG, JC, JMK and BZ designed experiments. EG and JC performed experiments. IM provided brain samples. JMK drafted the manuscript. All authors reviewed and edited the manuscript.

Competing interests

NRC, JMK, BZ, EG and JC received compensation from ProMIS Neurosciences. BZ and EG own stock options. JMK and NRC own common stock and stock options. IM has no competing interest.

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