Passive immunotherapy targeting amyloid-β reduces cerebral amyloid angiopathy and improves vascular reactivity

Kelly R. Bales,1 Sharon M. O’Neill,1 Nikolay Pozdnyakov,1 Feng Pan,1 David Caouette,1 YeQing Pi,1 Kathleen M. Wood,1 Dmitri Volfson,1 John R. Cirrito,2,3,4 Byung-Hee Han,5 Andrew W. Johnson,5 Gregory J. Zipfel2,3,5 and Tarek A. Samad1

Prominent cerebral amyloid angiopathy is often observed in the brains of elderly individuals and is almost universally found in patients with Alzheimer’s disease. Cerebral amyloid angiopathy is characterized by accumulation of the shorter amyloid-β isoform(s) (predominantly amyloid-β40) in the walls of leptomeningeal and cortical arterioles and is likely a contributory factor to vascular dysfunction leading to stroke and dementia in the elderly. We used transgenic mice with prominent cerebral amyloid angiopathy to investigate the ability of ponezumab, an anti-amyloid-β40 selective antibody, to attenuate amyloid-β accrual in cerebral vessels and to acutely restore vascular reactivity. Chronic administration of ponezumab to transgenic mice led to a significant reduction in amyloid and amyloid-β accumulation both in leptomeningeal and brain vessels when measured by intravital multiphoton imaging and immunohistochemistry. By enriching for cerebral vascular elements, we also measured a significant reduction in levels of soluble amyloid-β biochemically. We hypothesized that the reduction in vascular amyloid-β40 after ponezumab administration may reflect the ability of ponezumab to mobilize an interstitial fluid pool of amyloid-β40 in brain. Acutely, ponezumab triggered a significant and transient increase in interstitial fluid amyloid-β40 levels in old plaque-bearing transgenic mice but not in young animals. We also measured a beneficial effect on vascular reactivity following acute administration of ponezumab, even in vessels where there was a severe cerebral amyloid angiopathy burden. Taken together, the beneficial effects ponezumab administration has on reducing the rate of cerebral amyloid angiopathy deposition and restoring cerebral vascular health favours a mechanism that involves rapid removal and/or neutralization of amyloid-β species that may otherwise be detrimental to normal vessel function.

1 Pfizer Neuroscience and Pain Research Unit, 610 Main Street, Cambridge MA 02139, USA
2 Department of Neurology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA
3 Hope Center for Neurological Disorders, and Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA
4 Knight Alzheimer’s Disease Research Center, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA
5 Department of Neurological Surgery, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA

Correspondence to: Kelly R. Bales,
Pfizer Neuroscience and Pain Research Unit,
610 Main Street,
Cambridge MA 02139,
USA
E-mail: kelly.bales@pfizer.com
Introduction

Cerebral amyloid angiopathy (CAA) is characterized by the accumulation of amyloid-β peptide in the walls of leptomeningeal and cortical arteries and is thought to be causative to cerebrovascular disease affecting normal brain function especially cognition in the elderly (Vinters, 1987; Revesz et al., 2003; Arvanitakis et al., 2011). Indeed, CAA is almost always evident in brain tissue from subjects with sporadic late onset Alzheimer’s disease and also contributes to other brain injuries such as ischaemic stroke and intracerebral haemorrhage (Jellinger, 2002; Weller and Nicoll, 2003; Kimberly et al., 2009; Garcia-Alloza et al., 2011). In contrast to the parenchymal amyloid deposits, that are typically present in Alzheimer’s disease brain that are largely composed of the longer 42 amino acid amyloid-β fragment (amyloid-β42), cerebrovascular amyloid deposits associated with CAA are predominately composed of the shorter amyloid-β40 peptide (Ishii et al., 1997). Accumulation of amyloid-β40 in the cerebrovasculature and the subsequent appearance of CAA occur even when the primary source of amyloid-β is from neurons, as exemplified by several transgenic mouse models of CAA (Walker, 1997; Herzig et al., 2006). Critically, the level of parenchymal or vascular amyloid-β accumulation in these transgenic mouse models can be manipulated by altering the ratio of amyloid-β40:amyloid-β42 (Calhoun et al., 1999; Herzig et al., 2004, 2006). Additionally, age-dependent decline in clearance mechanisms typically efficient at removing amyloid-β peptides may contribute to further accumulation and damage (Manders et al., 2006). Recently, studies in humans have corroborated the hypothesis that inefficient amyloid-β clearance mechanisms may lead to abnormal amyloid-β accumulation in the brain (Mawuenyega et al., 2010).

Within individual cortical arteries, the accumulation of amyloid can be relatively heterogeneous with dense regions of CAA occurring adjacent to sections within the same vessel that are more sparsely affected. When mild-to-moderate amounts of CAA are present, the vessel is rendered unresponsive to normal physiological cues that require dynamic contraction and dilation in response to stimuli that may demand an increase in blood flow (Han et al., 2008). As more amyloid accretes, intercalation into the smooth muscle cell layer within the tunica media ensues, which when severe can lead to destruction of the smooth muscle cell layer, and compromise of the blood-brain barrier (Mandybur, 1986; Yamaguchi et al., 1992; Attens, 2005; Maia et al., 2007). Vessels that are severely affected by CAA may also take on a double-barreled appearance resulting in damage to the blood-brain barrier and extravasation of blood into the perivascular space. Both macro- as well as microhaemorrhages are associated with CAA and retention of the amyloid imaging agent Pittsburgh compound B (PiB) is often co-registered with microhaemorrhages when PET is used (Johnson et al., 2007; Ly et al., 2010). Additionally, CAA appears to accumulate more in posterior brain regions (Vinters and Gilbert, 1983). The functional consequence of CAA is reflected in diminished amplitude and time to peak in the blood oxygen level-dependent signal when measuring vascular reactivity in a visual task activated functional MRI paradigm (Smith et al., 2008; Dumas et al., 2012; Peca et al., 2013). In subjects with CAA the attenuated functional MRI response was attributed to impaired vasodilation that correlated with a greater number of microbleeds (Peca et al., 2013). Moreover, the reduction in vascular reactivity following a visual cue correlated to a greater volume of white matter hyperintensities suggesting that the blood oxygen level-dependent response to a visual cue may be an appropriate proxy for healthy neurovascular coupling (Iadecola, 2013; Peca et al., 2013).

While passive immunotherapy targeting various amyloid-β species has emerged as a potential therapeutic strategy for the treatment of Alzheimer’s disease, effects of these agents on removing vasculature amyloid-β load has not been as extensively investigated. Additionally, while passive immunization of transgenic mice with anti-amyloid-β antibodies has proven, in some studies, effective at removing parenchymal amyloid-β plaques, others have reported induction of microhaemorrhage even when anti-amyloid-β antibodies are administered to mouse models that have relatively sparse CAA pathology (Racke et al., 2005; Wilcock et al., 2006; Burbach et al., 2007). Previously we reported that chronic administration of ponezumab, a C-terminally directed anti-amyloid-β antibody to very old transgenic mice, which are also models of CAA, or cynomolgus monkeys did not result in an increase in brain microhaemorrhage even when very high doses were administered (Freeman et al., 2012a, b). Additionally there have been no clinical reports of vasogenic oedema, macro- or new haemorrhage in ponezumab treated subjects (Landen et al., 2013). Here we sought to investigate the efficacy of ponezumab to reduce or delay the accumulation of amyloid and amyloid-β in the cerebral vasculature using transgenic mouse models of Alzheimer’s disease that are also excellent models of CAA (Walker, 1997).
We hypothesized that peripheral ponezumab administration may effectively mobilize a brain interstitial fluid pool of amyloid-β₄₀, reducing vascular amyloid-β load and providing a beneficial effect on cerebrovascular function. Chronic administration of ponezumab to transgenic mice resulted in a significant reduction in the rate of CAA accumulation. Acute administration of ponezumab resulted in a significant improvement in the vasomotor response to various exogenous stimuli even in vessels with a significant CAA burden when compared to age-matched mice administered an irrelevant IgG. Taken together, the rapid and robust effects ponezumab treatment has on cerebral vascular reactivity in addition to the significant decrease in CAA following chronic administration favours a mechanism that involves rapid removal and/or neutralization of amyloid-β species that are otherwise detrimental to normal vessel function.

Materials and methods

Transgenic animals

All animal studies were conducted in accordance with the regulations and established guidelines for each of Institutional Animal Care and Use Committees. All mice were maintained in the vivarium under constant temperature and humidity on a 12:12 h light:dark cycle (lights on at 6 am) with *ad libitum* access to food and water. Both male and female PSAPP mice (PS1°C24/APPK670N/M671L, ~6 months old at the beginning of the study and maintained at Charles River Laboratories) were used for intravital multiphoton imaging studies and quantitative immunohistochemistry studies (Samaroo et al., 2012). For studies measuring vasodilatory responses female APP (APPK670N/M671L) transgenic mice at 12 months of age (Taconic) were used (Hsiao et al., 1996; Han et al., 2008). Ponezumab or an irrelevant IgG (Innovative Research) was administered via intraperitoneal (i.p.) injections. For chronic administration, ponezumab was administered weekly via intraperitoneal injections. Interstitial (i.p.) injections. For chronic administration, ponezumab was administered via intraperitoneal injections. Interstitial fluid levels of amyloid-β were measured in APP as well as APP PSdE9 (APPK670,M671L, PS1deltaE9, Jackson Laboratories) female transgenic mice at 18 and 12 months of age, respectively (Cirrito et al., 2003; Jankowsky et al., 2004). An additional cohort of young APP transgenic mice was also used to assess interstitial fluid levels of amyloid-β₄₀ following treatment.

Intravital multiphoton imaging

PSAPP mice (~6 months old at the beginning of the study) were used for intravital multiphoton imaging studies and were administered ponezumab (10 mg/kg, i.p) for 3 months. Briefly, PSAPP mice were injected with (10 mg/kg, i.p) methoxy X-034 to label amyloid 24–48 h before each imaging session. Mice were then deeply anaesthetized (80 mg/kg ketamine and 12 mg/kg xylazine) after which time the skull over the somatosensory cortex was thinned to ~20 μm in thickness using a dental drill (Pan and Gan, 2008). The brain vasculature was labelled following administration of 10 kDa rhodamine dextran (10 mg/kg intravenously) immediately before the animal was secured onto the stage of a multiphoton microscope. Images were captured using a two-photon laser that was mode-locked at 840 nm with a ×20 water immersion objective (Olympus, model number: XLUMPLFLN20XW, N.A = 1.00). The laser power at the sample sight was <40 mW. A z-stack image series was acquired from the pial surface to a depth of ~200 μm into the cortex. The same regions were imaged once every month. Each stack consisted of one colour channel to capture amyloid and one colour channel to capture the vascular structures. The image acquisition and data quantification were blinded with regard to the animal treatment paradigm. Image stacks were analysed using custom scripts in MATLAB (version 8.1.0, Mathworks Inc.) while post-processing and statistical analysis was performed in R (R Core Team, 2014). Arterial masks were drawn manually from maximum intensity projections, and were used as normalization factors to reduce variability across animals. We then fixed a threshold based on the intensity from overall histogram under each arterial mask calculated across all animals and time points. The number of positive voxels was used to quantify changes in plaque load across treatment groups and over time. We used a mixed model ANOVA with animal as a random factor to account for the random baseline intercept and fixed terms of treatment, time, and their interaction (Bates et al., 2013; Kuznetsova et al., 2013). This model was used to estimate planned pairwise comparisons across time points, and separately for each treatment, using least-squares means (Lenth, 2014). The Tukey-Kramer method was used to adjust for multiple hypothesis testing. All statistical tests were performed two-tailed at a 5% level of significance.

Isolation of brain vasculature

We enriched brain tissue for cerebrovascular elements with minor changes to previously published methods (Tontsch and Bauer, 1989; Supplementary Fig. 1). Briefly, after removing the cerebellum and olfactory bulb, the remaining brain was quickly frozen in liquid nitrogen and stored at −80°C until processed. Brain tissue was homogenized with a tight fitting pestle Dounce homogenizer in 3 ml of cold sucrose buffer (0.32 M sucrose, 5 mM HEPES, pH 7.4) (Tontsch and Bauer, 1989). The crude brain homogenate was centrifuged at 1000g for 10 min. and the supernatant containing mostly neuronal components was discarded. The dense white layer of myelin on the top of the pellet was also removed and discarded. The pellet was then resuspended in 3 ml of sucrose buffer and centrifuged at 1000g for 10 min. to remove any remaining myelin. An additional 40g spin was used to separate the large vessels from capillaries. The remaining pellet was washed an additional four times with 1 ml of sucrose buffer, and centrifuged at 350g for 10 min. Amyloid-β species were measured as described below following the addition of 20% sodium dodecyl sulphate (SDS) diluted in 0.32 M sucrose for a final concentration of 0.2% SDS. Enrichment for vascular elements in the final pellet was evaluated by western blot analysis probing for neuronal and endothelial markers. Additionally we used immunohistochemistry to visualize any remaining cells and the amyloid component.
Western blot analysis

Protein fractions from each of the cerebral vascular enrichment steps were size fractionated using gel electrophoresis (4–2% NuPAGE Bis-Tris or 10–20% Tris-Glycine gels), transferred onto nitrocellulose, and probed with the following primary antibodies directed against vascular or neuronal elements: Synaptophysin (Abcam ab52636); eNOS (BD610297); PECAM1/CD31 (Santa Cruz sc1506); pan Actin (Millipore MAB1501R); Glyceraldehyde phosphate dehydrogenase (Cell Signaling Technology 21185). An Odyssey Infrared Imager was used to visualize the signal.

Enzyme-linked immunosorbent assay

Measurement of amyloid-β peptides from samples enriched in cerebral vascular elements or crude brain homogenates was performed using a sensitive enzyme-linked immunosorbent assay (ELISA) (Lanz and Schachter, 2008). Briefly, homogenates and plasma were diluted with 5 M GuHCl or SDS following solid-phase extraction (Oasis HLB LP, 60 μm). Amyloid-β42 and amyloid-β40 were captured with an anti-amyloid-β42 (Rinat, 10G3) or amyloid-β40 (Rin, RN1219) selective antibodies. For assays measuring total amyloid-β, a N-terminal anti-amyloid-β antibody was used as the capture antibody (Covance; 6E10) followed by detection with a mid-domain anti-amyloid-β antibody (Covance; 4G8-Biotin). Amplification of Europium-Streptavidin signal was performed using time-resolved fluorescence of europium (excitation 340 nm, emission 615 nm) with an Envision multilabel plate reader. CSF was used directly in the amyloid-β total assay. Interstitial fluid amyloid-β-40 was measured in microdialysis samples by sandwich ELISA as previously described (Cirrito et al., 2003). The mouse-anti-amyloid-β40 antibody (mHJ2) was used as the capture antibody and biotinylated mouse-anti-amyloid-β (mHJ5.1) was used for the detection.

Immunohistochemistry

At the end of the study treatment period, PSAPP mice were perfused with phosphate-buffered saline (PBS), and the left hemisphere was placed into 10% neutral-buffered formalin for 24 h. The brain tissue was then processed in a TissueTek VIP 6 (Sakura Finetek) through graded alcohols to xylene. Finally, sections were counterstained with haematoxylin and passed from slides were then moved back to the Autostainer XL and sections were captured using a ScanScope XT (Aperio Technologies). The total number of vessels per section that were stained (50% coverage or greater) with anti-amyloid-β40 antibody or Congo Red were manually counted as positive by a rater blinded to the treatment paradigm. The number of vessels was plotted per section for each animal. Approximately six to nine sections per animal were quantified. A second rater blinded to treatment also quantified the amount of vascular amyloid-β40 antibody staining with results that were in direct agreement with the initial rater.

In vivo amyloid-β interstitial fluid measurements

The microdialysis procedure to measure the interstitial fluid levels of amyloid-β40 was performed as previously described (Ulrich et al., 2013). Briefly, APP (APPK670N/M671L, 18 months of age) or APP PseDe9 (APPK670N/M671L PS1deltaE9; 6 or 12 months of age) transgenic mice were stereotaxically implanted with the guide cannula tip (Eicom) at coordinates bregma −3.1 mm, 2.3 mm from midline 1.2 mm below the dura at a 12° angle. After surgery, mice recovered for 24 h followed by insertion of a 1000 kDa MWCO microdialysis probe that was 2 mm in length (AtmosLM probe, Eicom). Mice had freedom of movement and ad libitum food and water. Artificial CSF containing 4% bovine serum albumin was perfused through the microdialysis probe at a constant flow rate (inward rate = 1.1 μl/min; outward rate = 1.0 μl/min). Microdialysis samples were collected every 60–90 min in a refrigerated fraction collector. ‘Basal interstitial fluid amyloid-β concentration’ was defined as the mean concentration for the 9 h prior to initial drug treatment. Compound E, a potent γ-secretase inhibitor (200 nM; synthesized by AsisChem) was administered continuously to the brain by reverse microdialysis. After 24 h of Compound E, mice were administered ponezumab (10 mg/kg; i.p.) or an irrelevant IgG control and interstitial fluid amyloid-β was sampled for an additional 42 h. At the end of each study, all samples were analysed for amyloid-β-40. Following each experiment, probe placement was confirmed histologically.

Cerebral vasodilatory responses

Approximately 24 h prior to measurements of vasodilatory responses animals were randomly administered 10 mg/kg i.p. ponezumab or an irrelevant IgG isotype control (mouse IgG, Innovative Research). APP transgenic mice (APPK670N/M671L, n = 6 per group 12 months of age) were anaesthetized with isoflurane (5% for induction, 2% for maintenance). Core body temperature was maintained at 37°C by a thermoregulated heating pad (Cell MicroControls). The trachea was cannulated, and animals were ventilated mechanically (120 to 170 breaths per minute) with air and supplemental oxygen. A femoral arterial catheter was placed to measure mean arterial blood pressure and blood gases. A cranial window was made over the right parietal cortex, and a segment of a pial arteriole (i.e. the ramifications of the middle cerebral artery) was exposed by removing the overlying portion of dura mater with the tip of a 30-gauge hypodermic needle. Mice were then placed in a custom stereotactic frame and transferred to the microscope stage. Isoflurane anaesthesia was discontinued and replaced with α-chloralose (80 mg/kg, i.p.) to avoid isoflurane-induced vasodilation during vessel function assessment. The
cranial window was suffused at 20 μl/min with artificial CSF (temperature, 37 ± 0.5 °C; ionic composition (mM), NaCl (132), KCl (2.95), CaCl 2 (1.71), MgCl 2 (0.65), NaHCO 3 (24.6), d-glucose (3.69)) that was bubbled continuously with 83.5% N 2 /6.5 CO 2 /10% O 2 to produce the following levels in the cranial window: PCO 2 , 30.3 ± 1.2 mmHg; PO 2 , 164.7 ± 8.4 mmHg; and pH, 7.56 ± 0.02. The diameter of the exposed pial arteriole was recorded with a Nikon Eclipse 600ME microscope equipped with a microscopy system and MetaMorph imaging software (Molecular Devices). Pial arterioles having a diameter of ~20 μm were chosen to standardize the functional assessment across treatment groups. A blinded investigator then assessed cerebrovascular function by measuring changes in arteriolar diameter in response to acetylcholine (100 μM; Sigma Chemical Co.), S-nitro-N-acetyl-DL-penicillamine (SNAP, 500 μM, Sigma Chemical Co.), and hypercapnia induced by 5% carbon dioxide supplemented to supplied air by an operator that was blinded to treatment paradigm. The sequence of application of acetylcholine and SNAP to the cranial window was alternated between experiments. At least 15 min was allowed for vessel diameter to recover to control levels between application of vasodilator and/or condition. Just prior to vessel function analysis (normocapnia) and at the end of induced hypercapnia (hypercapnia), a sample of arterial blood was drawn into a capillary tube for measurement of blood gases, which were not significantly different across all groups tested (Supplementary Table 1). Vessel diameters were measured with ImageJ. Average vessel diameters across 25 μm longitudinal segments of pial arteries were analysed at baseline and after treatment with each vasodilatory stimulus. Data were calculated as per cent vasodilation versus baseline for each vessel diameter. To assess cerebral blood flow, a laser-Doppler probe was positioned stereotaxically on the parietal cortex though not to interfere with the light path of the microscopy system. All drugs were applied topically peripherally (weekly, 10 mg/kg, i.p., 5 months of age at study start) for 6 months, we quantified the amount of amyloid using Congo Red staining as well as amyloid-β 40 in cerebral vessels using immunohistochemistry. Following treatment with ponezumab, we quantified a significant decrease in the number of amyloid positive leptomeningeal and brain vessels with a greater decrease observed in the number of brain vessels that were amyloid positive (Fig. 2). Similarly, the number of amyloid-β 40 positive leptomeningeal and brain vessels following administration of ponezumab for 6 months were also significantly reduced (Fig. 2). In brain vessels the amount of amyloid-β 40 that we quantified in PSAPP treated mice was decreased nearly 50% following weekly administration of ponezumab for 6 months.

Isolation and quantification of amyloid-β from cerebral vessels following ponezumab treatment

Next we used a biochemical approach to measure amyloid-β peptides from brain tissue extracts that were enriched for vascular elements derived from the same animals used to quantify amyloid in individual vessels via intravital multiphoton microscopy (Supplementary Fig. 1). To ensure enrichment of vascular elements, we used western blot analysis to probe the relative level of proteins typically expressed in endothelial cells or neurons (Fig. 3). Additionally we used immunohistochemistry to visualize remaining cell types and amyloid-β/amyloid localization (Supplementary Fig. 1B and C). While a clear signal for synaptophysin was evident in the crude brain homogenate fractions, only PECAM1/CD31 and eNOS (encoded by Nos3) were evident in the fractions enriched for vascular elements (Fig. 3A). By enriching in cerebral vascular elements we were able to measure amyloid-β levels following treatment using a sensitive ELISA (Lanz and Schachter, 2008). Similar to the results we observed when quantifying the individual CAA vessel burden using intravital multiphoton imaging and immunohistochemistry, we also quantified a significant decrease in detergent soluble amyloid-β 40 and amyloid-β 42 levels in samples that were enriched for cerebral vascular elements (Fig. 3B and C). While there was also a decrease in the levels of both amyloid-β 40 and amyloid-β 42 (15–20%, respectively) in the insoluble guanidine fraction (Fig. 3D and E), the reduction did not reach significance. In the crude brain homogenate there was also a significant decrease in the levels of both amyloid-β 40 and amyloid-β 42 measured in the detergent soluble fraction following weekly administration of PSAPP mice with ponezumab for 3 months. The levels of both amyloid-β peptides in the insoluble guanidine brain extract were unchanged as was the amount of amyloid quantified when using immunohistochemistry (Fig. 4). There was no significant

Results

Reduction of cerebral amyloid angiopathy in PSAPP transgenic mice following ponezumab administration

We used intravital multiphoton microscopy of methoxy-XO34, an amyloid imaging agent, to quantify the development of CAA around cerebral vessels in living PSAPP (PS1 G384A, APP K670N,M671L) mice over time (Klung et al., 2002; Yang et al., 2010; Samaroo et al., 2012). At baseline, 6-month-old PSAPP mice had a significant amount of CAA in leptomeningeal vessels that increased significantly with age (Fig. 1). From 6 to 7 months of age there was a rapid and significant accumulation of CAA that continued over the ensuing 2 months but at a slower rate. When 6-month-old PSAPP transgenic mice were administered ponezumab peripherally (weekly, 10 mg/kg, i.p.) over the same period of time, there was no significant increase in the amount of CAA that accumulated (Fig. 1). In a separate cohort of PSAPP mice that were treated with ponezumab (weekly, 10 mg/kg, i.p., 5 months of age at study start) for 6 months, we quantified the amount of amyloid using Congo Red staining as well as amyloid-β 40 in cerebral vessels using immunohistochemistry. Following treatment with ponezumab, we quantified a significant decrease in the number of amyloid positive leptomeningeal and brain vessels with a greater decrease observed in the number of brain vessels that were amyloid positive (Fig. 2). Similarly, the number of amyloid-β 40 positive leptomeningeal and brain vessels following administration of ponezumab for 6 months were also significantly reduced (Fig. 2). In brain vessels the amount of amyloid-β 40 that we quantified in PSAPP treated mice was decreased nearly 50% following weekly administration of ponezumab for 6 months.
Mobilization of interstitial fluid amyloid-β_{40} following ponezumab administration

A direct correlation between plasma and brain amyloid-β levels was measured following peripheral administration of an anti-amyloid-β antibody to plaque bearing transgenic mice (DeMattos et al., 2002). The robust change in peripheral levels of amyloid-β that were measured after anti-amyloid-β antibody administration could be due, at least in part, to the mobilization of amyloid-β that may be sequestered in the periphery. Here we directly measured brain interstitial fluid levels of amyloid-β\textsubscript{40} to determine if there would be a pool of amyloid-β that could be mobilized after peripheral administration of ponezumab (Cirrito et al., 2003). We combined reverse microdialysis using a potent gamma secretase inhibitor to inhibit de novo synthesis of amyloid-β with peripheral administration of ponezumab and measured interstitial fluid amyloid-β\textsubscript{40} levels in both old and young transgenic mice. We hypothesized that in older plaque bearing mice, infusion of a gamma secretase inhibitor would block de novo synthesis of amyloid-β and reveal a pool of amyloid-β that was both responsive to ponezumab administration and reflective of deposited material in brain. As previously reported, in both young and old (6 and 18 months of age) APP (\textit{APP}K\textsubscript{670N,M671L}) transgenic mice infusion of a gamma secretase inhibitor resulted in a significant decrease in interstitial fluid amyloid-β\textsubscript{40} levels (Hsiao et al., 1996; Cirrito et al., 2003). Following a single administration of ponezumab to older plaque bearing transgenic mice, interstitial fluid levels of amyloid-β\textsubscript{40} increased dramatically (Fig. 5B). The significant increase in interstitial fluid amyloid-β\textsubscript{40} levels was sustained for ~24 h after ponezumab administration. To ensure that peripheral ponezumab was indeed mobilizing a soluble pool of amyloid-β\textsubscript{40} derived from brain and not the result of efflux of an amyloid-β:antibody complex from plasma back into brain, we measured interstitial fluid amyloid-β\textsubscript{40} levels in young APP transgenic mice devoid of any detectable parenchymal plaques using the same experimental paradigm. As expected, following infusion of a gamma secretase inhibitor via reverse microdialysis, interstitial fluid amyloid-β\textsubscript{40} levels dropped dramatically in young APP transgenic mice. After acute administration of ponezumab, the levels of interstitial fluid amyloid-β\textsubscript{40} remained low (Fig. 5A). As brain amyloid-β load varies across transgenic mouse strains, we next used an additional line of transgenic mice; APP/PS1dE9 (\textit{APP}K\textsubscript{670N,M671L}).
PS1deltaE9), to complement and extend our initial observations (Jankowsky et al., 2004). Again, in plaque bearing APP/PS1dE9 mice, interstitial fluid amyloid-β40 levels dropped dramatically following reverse microdialysis of a gamma secretase inhibitor and then significantly increased following administration of ponezumab (Fig. 5B). To determine if the increase in interstitial fluid amyloid-β40 that was mobilized following acute ponezumab administration was

Figure 2 Quantification of amyloid-β and amyloid in cerebral vessels following administration of ponezumab. Significant reduction in Congo red-positive amyloid in leptomeningeal (A) and brain vessels (B). Significant reduction in the number of leptomeningeal (C) or brain vessels (D) immunoreactive for amyloid-β40 following administration of ponezumab. PSAPP mice (6 months of age at study start) were treated for 6 months with ponezumab (10 mg/kg; weekly intraperitoneal; n = 6–9 mice per group). The number of vessels (average number/section/mouse) that were stained (>50% coverage) were plotted for each section analysed (6–9 sections analysed per animal). Data are represented as mean ± SEM. *P < 0.05, **P < 0.01. Representative lower power image of mid (E) and rostral (F) sections stained with anti-amyloid-β40 antibody (Scale bar = 500 μm) from vehicle treated group. High power surface (e) and parenchymal (f) vessel (Scale bar 25 μm).
directly related to the amount of amyloid around the dialysis probe, we quantified amyloid burden, both parenchymal and vascular, in the hippocampal region that was proximal to the location of the probe. There was a significant correlation between hippocampal plaque load around the microdialysis probe and the increase over basal interstitial fluid amyloid-\(\beta\) \(40\) levels after ponezumab administration in both the APP and APP/PS1dE9 transgenic lines that we used (Fig. 5C). Even when ponezumab was administered alone, without infusion of a gamma secretase inhibitor, there was a significant increase in interstitial fluid amyloid-\(\beta\) \(40\) levels with the initial rate of increase nearly identical between groups (Supplementary Fig. 7). Taken together, these results suggest that peripheral ponezumab administration mobilizes an interstitial fluid pool of amyloid-\(\beta\) \(40\) that is present in old plaque bearing transgenic mice only.

**Reversal of vascular reactivity impairment following ponezumab administration**

Previously we demonstrated that vascular reactivity in response to acetylcholine and hypercapnia was altered in APP transgenic mice (Han *et al.*, 2008). To determine if acute ponezumab administration could rescue the vasomotor impairments observed in APP transgenic mice, we measured vascular reactivity in individual cortical vessels in APP transgenic mice following a challenge with various pharmacological and physiological agents known to induce vasodilation. The vascular response following various challenges was significantly blunted in APP transgenic mice when compared to similarly aged wild-type mice (Fig. 6) while all other physiological parameters were equivalent between groups (Supplementary Table 1). In old APP transgenic mice a single administration of ponezumab \(24\) h prior to application of acetylcholine resulted in a significant and robust improvement in the vasodilatory response (Fig. 6A and B). The vasodilatory responses to hypercapnia or \(\text{S-nitro}-\text{N-acetyl-DL-penicillamine}\) (SNAP) were also significantly increased (Fig. 6C and D). To study the relationship between CAA burden and the blunted vasodilatory response, we quantified the amount of CAA in \(25\) \(\mu\)m vessel segments in old transgenic mice and measured the dilatory response to acetylcholine in individual vessels with varying levels of CAA (0–20%, 21–60%, and 61–100%). Ponezumab administration resulted in a significant increase in the vasomotor response to acetylcholine regardless of CAA burden and even in vessel segments that had maximal CAA coverage (61–100%) (Fig. 7). We also probed whether or not vessel integrity was compromised in...
anyway by quantifying the number of microhaemorrhages following chronic ponezumab administration. After 6 months of ponezumab administration there was a slight decrease in the total number of brain microhaemorrhages that were quantified by Prussian blue staining, which did not reach statistical significance (Supplementary Fig. 8).

Discussion

CAA is a significant age-dependent risk factor for ischaemic and haemorrhagic stroke and likely contributes to vascular dysfunction leading to dementia. The first identification of amyloid-β peptide occurred through isolation and amino acid sequencing of Congo Red-positive tissue samples enriched in cerebral vasculature elements (Glenner and Wong, 1984). This initial observation was later extended to sporadic late onset forms of Alzheimer’s disease confirming that a major component of neuritic plaque is also composed of amyloid-β peptides though, in contrast to CAA, the amyloid-β42 species is more predominant. Although rare, some heritable forms of CAA occur when mutations within the amyloid-β sequence result in peptide accumulation on the brain side of the cerebral vasculature which likely occurs as a direct result of mutation-dependent inefficiency of amyloid-β egress from brain (Monro et al., 2002; Maat-Schieman et al., 2005). While CAA results in a variable neuropathological distribution, the most common sites for accumulation occur in vessels of the neocortex and leptomeninges and most frequently in the tunica media and adventitia of arterioles (Jellinger, 2002).

Ultrastructural vascular changes in transgenic animal models of CAA include damage to endothelial and smooth muscle cells even in the absence of quantifiable amyloid deposits suggesting that the more soluble forms of amyloid-β are also detrimental to normal vascular function (Han et al., 2008). Thickening of the basement membrane, pericyte degeneration as well as branching and segmentation of vessels are additional pathological features of CAA. Abnormal segmentation and branching may eventually result in weakening of vessels and increased susceptibility to shear force damage.

Emerging evidence suggests that impairment in the clearance of amyloid-β from brain may contribute to the accumulation of amyloid-β affecting brain regions that are critical for learning and memory. Indeed, the rate of de novo production of amyloid-β40 in CSF from humans measured using a sensitive stable isotope labelling kinetic technique is quite high (~400 ng/h) necessitating efficient in situ clearance mechanisms to avoid parenchymal deposition. While catalytic enzyme systems such as neprilysin and insulin degrading enzyme exist in brain to remove neuronal derived amyloid-β species, additional amyloid-β transport pathways also exist. Direct transport of amyloid-β out of the brain occurs via the lipoprotein-related protein receptor in an apolipoprotein E-dependent fashion as well as removal via the peri-arterial drainage system to the CSF compartments. Tracer studies have also identified interstitial fluid drainage as an egress system in grey matter that then merges into bulk flow pathways (Nicholson et al., 2000; Kimberly et al., 2009). The movement of solutes and fluid thus drains along arterial walls perhaps even requiring the pulsatile nature of these structures to

Figure 4 Reduction in detergent soluble amyloid-β levels in brain. Significant decrease in detergent soluble amyloid-β40 (A) and amyloid-β42 (B) levels that were extracted from brain tissue of PSAPP mice after weekly administration of ponezumab for 6 months (6 months of age at study start; 10 mg/kg, weekly intraperitoneal administration) There was no significant reduction in amyloid-β40 (C) or amyloid-β42 (D) in the guanidine extractable pool from brain homogenates or when quantifying the number of amyloid-β40 (E) or amyloid (thioflavin S) positive plaques (F) parenchymal plaques. Representative image (median) of parenchymal plaque-laden piriform cortex demonstrating amyloid-β40 immunoreactivity from vehicle (G) or ponezumab treated (H). Data are represented as mean ± SEM.*P < 0.05, **P < 0.01, n = 6–9 per group. Scale bar = 100 μm.
maintain an appropriate level of flow. During normal aging, many of the amyloid-β transport systems appear to lose efficiency contributing to an environment proximal to the cerebral vascular egress pathways that is more conducive for amyloid-β/amyloid accumulation and subsequent deposition. For instance, the levels of neprilysin, a very efficient catalytic enzyme known to degrade amyloid-β are reduced in an age- and apolipoprotein E-dependent fashion in cerebral arteries (Iwata et al., 2000; Miners et al., 2006). Indeed, a significant reduction in CSF amyloid-β40 levels was reported to occur in asymptomatic subjects with CAA suggesting that like reduced CSF amyloid-β42 levels measured in subjects with Alzheimer’s disease with significant brain amyloid burden, reduced CSF amyloid-β40 levels may inform the level of CAA burden (Blennow and Hampel, 2003; Verbeek et al., 2009).

Given the requirement for brain to have multiple mechanisms for efficient removal of brain derived amyloid-β, we investigated the ability of peripherally administered ponezumab, an antibody directed towards the C-terminus of amyloid-β, to attenuate the accumulation of amyloid-β in cerebral vessels in several lines of transgenic mice that are models of CAA. In addition to an age-dependent accrual of amyloid-β in brain, these transgenic mice also accumulate significant levels of CAA and have been useful models to interrogate the molecular mechanisms associated with CAA. We utilized the murine version of a C-terminally directed anti-amyloid-β antibody, ponezumab, to investigate both chronic as well as acute effects after administration to several transgenic mouse models of CAA (Freeman et al., 2012). Ponezumab has high affinity and selectivity towards amyloid-β40 in addition to engineered mutations for efficient removal of brain derived amyloid-β.

Figure 5 Interstitial fluid levels of amyloid-β40. Following infusion of a potent gamma secretase inhibitor (Cmpd E; 200 nM; GSI) and peripheral administration of ponezumab (10 mg/kg; intraperitoneal) to APP transgenic mice there was a significant increase in interstitial fluid amyloid-β levels in old plaque bearing mice only (A). Quantification of the change from basal interstitial fluid (ISF) levels over a 30.5–44-h time period after gamma secretase inhibitor infusion and ponezumab administration (B). There is a significant correlation between basal interstitial fluid amyloid-β40 levels and total hippocampal plaque load (both parenchymal and vascular) proximal to the microdialysis probe following treatment (C). (APP = APPK670N;M671L; old/young = 18/6 months of age; APP/PS1 = APPK670N;M671L/PS1deltaE9; 12 months of age), n = 3–6 per group (Cirrito et al., 2003).
within the Fc region to reduce immune effector function (La Porte et al., 2012). Chronic administration of ponezumab to PSAPP mice resulted in a significant reduction in CAA accumulation assessed both by histology and via quantitative intravital multiphoton microscopy of individual cerebral vessels. The age-dependent reduction in amyloid accumulation was also reflected in our biochemical measurements of amyloid-β using tissue that was enriched for cerebral vascular elements and a sensitive ELISA that measured various amyloid-β peptides. Interestingly in addition to a significant decrease in the level of detergent soluble amyloid-β40, we also quantified a significant decrease in the level of amyloid-β42 following administration of ponezumab for 3 months. While the levels of the less mobilizable and insoluble pool represented by the guanidine extractable amyloid-β species were decreased, the reduction did not reach significance (for amyloid-β42 $P = 0.0515$). In a separate cohort of PSAPP mice we confirmed our multiphoton and biochemical measurements by quantifying a significant reduction in the number of vessels that were positive for amyloid or amyloid-β40 by immunohistochemistry. There was no significant change in the levels of amyloid-β measured in brain homogenates or parenchyma using immunohistochemistry suggesting that the significant reduction in detergent soluble amyloid-β species that we measured in our vascular enriched fraction was not the result of contamination from parenchymal deposits. Additionally, if there was an unavoidable contamination of brain parenchyma amyloid-β into our vascular preparation we would have measured an increase and not a decrease in the relative levels. While our studies may have been underpowered to detect a robust decrease in parenchymal amyloid-β load we would anticipate that a longer treatment period would have a more profound effect on this particular biochemical pool of amyloid-β. Taken together, our results demonstrate that chronic administration of ponezumab to transgenic mice with considerable CAA at the beginning of the treatment paradigm results in a significant reduction in the rate of CAA accumulation. Furthermore, biochemical analysis of tissue enriched in cerebral vascular elements suggests that the significant reduction in amyloid-β40 specific accumulation may also have beneficial effects on the removal of amyloid-β42. While additional studies will be required to more fully understand this latter observation, it is interesting to speculate that by enhancing the removal of amyloid-β40 from select vascular egress highways, amyloid-β42 removal may also occur. Our biochemical methods did not allow us to characterize the potential for ponezumab administration to capture and remove heterogeneous species of amyloid-β that may also be composed of amyloid-β40:amyloid-β42 dimers. Ponezumab administration was also selective for the reduction of amyloid-β/amyloid in the vasculature since brain parenchymal levels were unchanged. As previously reported we observed a transgene-dependent attenuation in vasomotor responses in APP (APP $^{K670N;M671L}$) transgenic mice. Even at a young age when soluble levels of amyloid-β are high but CAA is absent, APP transgenic mice have severely impaired responses to the endothelium-dependent dilator acetylcholine (Han et al., 2008). Additionally, the vasomotor responses to hypercapnia and SNAP were also significantly impaired. These stimuli are anticipated to induce physiological and

**Figure 6 Experimental paradigm and vascular reactivity measurements.** Ponezumab (10 mg/kg, intraperitoneal) or an irrelevant IgG (IgG) was administered ~24 h prior to baseline measurements followed by exogenous application of vasodilators: acetylcholine (Ach), S-nitro-N-acetyl-DL-penicillamine (SNAP), or hypercapnia (A). Prior administration of ponezumab results in a significant increase in vasodilatation following exposure to acetylcholine (B), SNAP (C) or hypercapnia (D). There was no significant effect following administration of an equivalent dose of an irrelevant IgG. APP = APP $^{K670N;M671L}$; 12 months of age (37). Data are represented as mean ± SEM, *$P < 0.05$, **$P < 0.01$ versus APP IgG, $n = 6–7$. 

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Figure 7 Dilatory response (%) of individual vessels. APP transgenic mice with various levels of CAA burden following acute administration of ponezumab or an irrelevant IgG (10 mg/kg, intraperitoneal) (A). Representative individual vessels with various levels of CAA burden stained with methoxy X034 (X04), at baseline or after exogenous acetylcholine exposure (+Ach) and following acute ponezumab (Pon) or irrelevant IgG administration (10 mg/kg, intraperitoneal) (B). n = 6–7 animals per group APP = APPK670N,M671L at 12 months of age.
endothelial-independent responses, respectively, suggesting that cerebrovascular impairment by more soluble forms of amyloid-\(\beta\) may involve several mechanisms. In APP transgenic mice with CAA, the vasomotor responses to all of the stimuli tested were substantially improved following administration of ponezumab although the extent of rescue was incomplete when compared to similarly aged wild-type mice. Even when the CAA burden within individual vessels was quite high (61–100\%), acute ponezumab administration resulted in a significant vasomotor response to acetylcholine. Cerebral blood flow measurements were similar across all of the groups tested suggesting that the robust effects of acute ponezumab administration on vessel reactivity in APP transgenic mice were likely pleiotropic affecting multiple cell types important for efficient neurovascular coupling. While the effects of acute ponezumab administration were robust and occurred without altering the level of CAA burden, the magnitude of restoration may still be insufficient and further studies will be required to more fully explore this complexity.

The toxic actions of amyloid-\(\beta_{40}\) on cerebral vascular cells are well characterized (Paris et al., 2003). More complexity arises, however, when hypotheses are proposed for the dynamic egress of amyloid-\(\beta\) from the human brain especially when parenchymal deposits appear to be resolved. Recent data, however, supports the working hypothesis that brain derived amyloid-\(\beta\) species may indeed be removed via peripheral anti-amyloid-\(\beta\) antibody administration at least in transgenic mouse model systems. While the exact mechanism whereby certain peripherally administered anti-amyloid-\(\beta\) antibodies can exert this effect are not well understood, we used measurements of interstitial fluid amyloid-\(\beta\) levels in plaque bearing APP transgenic mice to better understand these complicated dynamics (Cirrito et al., 2003). As expected, when \textit{de novo} synthesis of amyloid-\(\beta\) was inhibited by applying a well characterized gamma secretase inhibitor, interstitial fluid levels of amyloid-\(\beta\) decreased dramatically. This latter result occurred in both young and old mice. To interrogate whether or not a pool of amyloid-\(\beta\) in brain parenchyma was available that could be mobilized, we administered ponezumab peripherally. In old plaque bearing mice but not in young mice, we observed a robust and significant increase in the level of amyloid-\(\beta_{40}\) that we were able to measure in interstitial fluid samples following ponezumab administration. The increase lasted for \(>48\) h before returning to baseline levels. While further investigation will be required, it is reasonable to speculate that mobilization of this interstitial fluid pool of amyloid-\(\beta_{40}\) is directly related to a beneficial effect on vessel reactivity, as we measured an improvement (rather than a diminution) in vascular reactivity. In that regard, we also measured a robust and significant correlation between the amount of amyloid directly proximal to the locale of the microdialysis probe and the change from baseline in the levels of interstitial fluid amyloid-\(\beta_{40}\) after ponezumab administration suggesting that this mobilizable pool is associated with parenchymal deposits either directly and/or loosely via a concentration gradient. The effects of a single administration of ponezumab were long lasting as interstitial fluid amyloid-\(\beta_{40}\) levels remained elevated even in animals that did not receive infusion of a gamma secretase inhibitor. The ponezumab-dependent mobilization of interstitial fluid amyloid-\(\beta_{40}\) may also provide insight into the mechanism by which chronic treatment leads to a decrease in vascular amyloid since recent studies have suggested a vascular origin of parenchymal amyloid-\(\beta\) deposits (Kumar-Singh et al., 2005).

Ponezumab is a C-terminally selective anti-amyloid-\(\beta\) antibody with high affinity towards amyloid-\(\beta_{40}\) that has been engineered to minimize Fc effector function and complement activation (La Porte et al., 2012). These attributes likely contribute to the ability of ponezumab to bind to more soluble forms of amyloid-\(\beta_{40}\) without causing an increase in the incidence of microhaemorrhage. Indeed, ponezumab was well tolerated and did not increase the incidence of microhaemorrhage when high doses were administered to very old transgenic mice or cynomolgus monkeys chronically (Freeman et al., 2012a, b). Again, in our current studies we did not observe any significant increase in the number of microhaemorrhages but rather a slight decrease suggesting that vessel integrity may even improve following chronic ponezumab administration. Prior studies using an anti-amyloid-\(\beta_{40}\) selective antibody in old APP mice resulted in an increase in CAA and microhaemorrhage even when the effector function was dampened biochemically suggesting Fc effector function may be causative to the appearance of microhaemorrhage (Wilcock et al., 2006). By introducing mutations into the Fc region of ponezumab the effector function and complement activation were essentially eliminated thereby minimizing any potential to induce microhaemorrhage. Epitope specificity is also important for minimizing subsequent microhaemorrhage and anti-amyloid-\(\beta\) antibodies directed towards the N-terminus of amyloid-\(\beta\) appear to be particularly susceptible for causing these events even in humans (Pfeifer et al., 2002; Racke et al., 2005; Sperling et al., 2012).

Disappointingly, various anti-amyloid-\(\beta\) immunotherapies have not demonstrated clinical efficacy in subjects with Alzheimer’s disease who are diagnosed with mild to moderate disease in recently completed phase 3 trials (Salloway et al., 2014a, b). Given the heterogeneous aetiology of Alzheimer’s disease and the probability that in mild/moderate subjects with Alzheimer’s disease co-morbid neuro-pathologies may limit any potential clinical effect requires further refinement of the Alzheimer’s disease continuum diagnosis and careful consideration of subject inclusion. In this regard, clinical trials have now been initiated in populations that are ‘at risk’ or in individuals who also have a biomarker signature indicative of Alzheimer’s disease in an effort to more appropriately measure efficacy (Sperling et al., 2014). Emerging evidence also suggests that accumulation of amyloid-\(\beta\) peptide in its various forms in brain, while necessary, may not be sufficient to...
cause cognitive decline in all individuals. Taken together our data provide a greater appreciation for cerebrovascular dysfunction caused by amyloid-β and the ability of ponezumab to neutralize, at least in animal models, some of these effects meriting further clinical investigation in populations predisposed to develop CAA and also patients with Alzheimer’s disease.

Acknowledgements

We would like to thank Cathy Gonzales and the Comparative Medicine staff of Pfizer, Inc. for their care and attention to the live-phase experimentation section of this study. Additionally, we would like to thank Drs John Lin, Rita Balice-Gordon, and David Riddell for critical review and helpful suggestions to this manuscript. K.R.B., S.M.O’N., N.P., F.P., D.C., Y.P., K.M.W., D.V., and T.A.S. are or were full-time employees of Pfizer, Inc. at the time the studies were conducted. F.P. completed multiphoton studies. S.M.O’N., D.C, N.P., Y.P., K.M.W., and D.V. completed immunohistochemistry and biochemistry studies. J.R.C. completed interstitial fluid studies, B. H.-H., A.J., and G.Z. conceived and completed vascular reactivity studies. K.R.B. and T.A.S. conceived of the experimental paradigms and wrote the manuscript. All authors commented on the final manuscript submission.

Funding

All studies were funded by Pfizer, Inc.

Supplementary material

Supplementary material is available at Brain online.

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Klüver WE, Bacska BJ, Mathis CA, Kajdasz ST, McLellan ME, Frosch MP, et al. Imaging Abeta plaques in living transgenic mice with multiphoton microscopy and methoxy-X04, a systematically


