Elucidating the Aβ42 Anti-Aggregation Mechanism of Action of Tramiprosate in Alzheimer’s Disease: Integrating Molecular Analytical Methods, Pharmacokinetic and Clinical Data

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Abstract

Background Amyloid beta (Aβ) oligomers play a critical role in the pathogenesis of Alzheimer’s disease (AD) and represent a promising target for drug development. Tramiprosate is a small-molecule Aβ anti-aggregation agent that was evaluated in phase III clinical trials for AD but did not meet the primary efficacy endpoints; however, a pre-specified subgroup analysis revealed robust, sustained, and clinically meaningful cognitive and functional effects in patients with AD homozygous for the ε4 allele of apolipoprotein E4 (APOE4/4 homozygotes), who carry an increased risk for the disease. Therefore, to build on this important efficacy attribute and to further improve its pharmaceutical properties, we have developed a prodrug of tramiprosate ALZ-801 that is in advanced stages of clinical development. To elucidate how tramiprosate works, we investigated its molecular mechanism of action (MOA) and the translation to observed clinical outcomes.

Objective The two main objectives of this research were to (1) elucidate and characterize the MOA of tramiprosate via an integrated application of three independent molecular methodologies and (2) present an integrated translational analysis that links the MOA, conformation of the target, stoichiometry, and pharmacokinetic dose exposure to the observed clinical outcome in APOE4/4 homozygote subjects.

Method We used three molecular analytical methods—ion mobility spectrometry–mass spectrometry (IMS–MS), nuclear magnetic resonance (NMR), and molecular dynamics—to characterize the concentration-related interactions of tramiprosate versus Aβ42 monomers and the resultant conformational alterations affecting aggregation into oligomers. The molecular stoichiometry of the tramiprosate versus Aβ42 interaction was further analyzed in the context of clinical pharmacokinetic dose exposure and central nervous system Aβ42 levels (i.e., pharmacokinetic–pharmacodynamic translation in humans).

Results We observed a multi-ligand interaction of tramiprosate with monomeric Aβ42, which differs from the traditional 1:1 binding. This resulted in the stabilization of Aβ42 monomers and inhibition of oligomer formation and elongation, as demonstrated by IMS–MS and molecular dynamics. Using NMR spectroscopy and molecular dynamics, we also showed that tramiprosate bound to Lys16, Lys28, and Asp23, the key amino acid side chains of Aβ42 that are responsible for both conformational seed formation and neuronal toxicity. The projected molar excess of tramiprosate versus Aβ42 in humans using the dose effective in patients with AD aligned with the molecular stoichiometry of the interaction, providing a clear clinical translation of the MOA. A consistent alignment of these preclinical-to-clinical elements describes a unique example of translational medicine and supports the efficacy seen in symptomatic patients with AD. This unique “enveloping mechanism” of tramiprosate also provides a potential basis for tramiprosate dose selection for patients with homozygous AD at earlier stages of disease.
Conclusion We have identified the molecular mechanism that may account for the observed clinical efficacy of tramiprosate in patients with APOE4/4 homozygous AD. In addition, the integrated application of the molecular methodologies (i.e., IMS-MS, NMR, and thermodynamics analysis) indicates that it is feasible to modulate and control the Aβ42 conformational dynamics landscape by a small molecule, resulting in a favorable Aβ42 conformational change that leads to a clinically relevant amyloid anti-aggregation effect and inhibition of oligomer formation. This novel enveloping MOA of tramiprosate has potential utility in the development of disease-modifying therapies for AD and other neurodegenerative diseases caused by misfolded proteins.

Key Points

| We have elucidated and characterized the molecular mechanism of action of tramiprosate. |
| Tramiprosate modulates conformational flexibility of amyloid beta Aβ42, leading to the prevention of oligomer seed formation and thus aggregation. |
| Translational analysis shows an alignment of the three described molecular effects of Aβ42 with pharmacokinetic and published clinical data. |

1 Introduction

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disorder, affecting a large number of elderly people worldwide. It is widely accepted that amyloid beta (Aβ) is one of the key pathogenic causes for AD [1, 2]. The level of soluble, non-fibrillar Aβ oligomers in the brain correlates strongly with the severity of the disease [1, 3, 4], suggesting that soluble oligomeric species of Aβ, rather than the fibrillar form within amyloid plaques, likely play a pivotal role in AD pathophysiology.

Aβ peptides, particularly Aβ42, have a strong intrinsic tendency to self-assemble and form aggregates that constitute neurotoxic oligomeric species [5]. Monomeric Aβ peptides exhibit very high conformational flexibility [6, 7], which represents one of the major challenges for this therapeutic target. The initial random coil structure shows characteristics of an α-helix and β-sheet mixture that transforms into a final structure predominantly comprising β-sheets [8]. At this stage, a nucleation phase occurs that initiates Aβ aggregation. Soluble Aβ oligomers have been shown to form by a nucleation-dependent process, wherein most neurotoxic Aβ42 becomes a seed in the aggregation process and also enhances the oligomerization of Aβ40, the most prevalent species of Aβ in the central nervous system (CNS) [9]. The conformational state of Aβ42 thus plays a critical role in the formation of oligomers, especially in the formation of initiation seeds for Aβ aggregation.

In this study, we focused on tramiprosate, 3-amino-propanesulfonic acid, a homolog of the amino acid taurine, that has been shown in preclinical studies to bind to soluble Aβ and inhibit Aβ aggregation, leading to a reduction of Aβ plaque load [10] (Fig. 1). The safety and efficacy of tramiprosate has been evaluated in the nonclinical program and in 16 clinical trials, including two phase III trials in over 2000 subjects with AD. Across these studies, tramiprosate safety and tolerability were favorable, and the main adverse events were nausea and vomiting, which are being addressed by the development of tramiprosate prodrug ALZ-801. While the phase III studies in patients with mild-to-moderate AD did not meet the primary efficacy endpoints, a pre-specified subgroup analysis revealed robust, sustained, and clinically meaningful cognitive and functional effects in patients with AD homozygous for the ε4 allele of apolipoprotein E4 (APOE4/4 homozygotes) [11]. Importantly, there was evidence of an APOE4 gene dose effect, where the order of efficacy on both cognition and function followed the number of APOE4 alleles. The APOE4/4 homozygotes showed larger (almost double) efficacy than APOE4 heterozygotes, and the APOE4 heterozygotes showed higher efficacy than non-carriers. This APOE4 gene-dose effect likely reflects the fact that APOE4 carriers have a higher burden of amyloid pathology, with APOE4/4 homozygotes thought to have the highest burden of Aβ oligomer pathology. To date, the mechanism of action (MOA) for tramiprosate and the nature of its molecular interactions with Aβ peptides, has not been elucidated.

Fig. 1 Chemical structure of tramiprosate (left) and amino acid sequence of amyloid beta Aβ42 (right)
2 Objectives

The two main objectives of this research were to (1) elucidate and characterize the molecular MOA of tramiprosate via an integrated application of three independent molecular methodologies—ion mobility spectrometry–mass spectrometry (IMS–MS), nuclear magnetic resonance (NMR), and thermodynamics analysis—and (2) present an integrated translational analysis that links the MOA, conformation of the target, stoichiometry, and pharmacokinetic dose exposure to the observed clinical outcome in APOE4/4 homozygote subjects. We hereby report the discovery and elucidation of a novel multi-ligand enveloping MOA, which mediates the Aβ anti-aggregation activity of tramiprosate and potentially underpins its clinical efficacy in patients with amyloid-bearing AD (Fig. 2). A number of intertwining molecular aspects of this MOA form a coherent understanding of the control of conformational flexibility of Aβ and its impact on clinical outcome. The integrated translational analysis shows a consistent alignment of these preclinical-to-clinical elements, describing a unique example of translational medicine.

3 Methods

3.1 Molecular Modeling and Molecular Dynamics Simulations

All molecular modeling was performed using the Schrödinger suite (2015-3; Schrödinger, LLC; New York, NY, USA; 2015). Molecular dynamics simulations were run using Desmond [12]. The simulations were run on GeForce GTX Titan Black graphics processing unit cards. The optimized potential for liquid simulations (OPLS 3.0) force field [13] was used to model all interactions, and the SPC model was used for waters. The 1IYT Aβ42 NMR structure from the Protein Data Bank (PDB) was used as a starting point for molecular dynamics simulations. This structure is primarily alpha helical and is representative of the peptide in an apolar environment. A 20-Å box of water or a mixed solvent box of 1 % tramiprosate in water was added around the peptide using Schrödinger system set-up tools. Ions were added to neutralize the charge of the entire system. Simulations were equilibrated and run under NPT conditions [constant number (N), pressure (P) and temperature (T)] with periodic boundary conditions. A Nose–Hoover Thermostat and Martina–Tobias–Klein barostat were used to control temperature and pressure, respectively. Simulations were run in replicates of three for 100 ns each, and the results were compiled for analysis. Principal component (PC) analysis was performed using ProDy [14] and plotted using custom python scripts.

3.2 Ion Mobility Spectrometry–Mass Spectrometry (IMS–MS)

The conditions used for MS, using a Waters Synapt G2-S, were as follows: positive polarity in sensitivity mode; capillary = 2.5 kV; nebulizer = 2 mbar; source temperature = 80 °C; desolvation temperature = 60 °C; sample cone setting = 35 V; source offset setting = 60 V; and
We reconstituted 1 mg of recombinant human Aβ3.2.1 Sample Preparation

conducted at Protea, Inc. (Morgantown, WV, USA). infused at room temperature. The IMS–MS studies were the detection of the less abundant oligomers. Samples were acquired using the systems sensitivity mode to allow for the acquisition of the amyloid peptide was performed using a Waters Synapt G2-S quadrupole time of flight mass spectrometer (Q-TOF MS) with traveling wave ion mobility (Waters Corp., Milford, MA, USA). The data were acquired using the systems sensitivity mode to allow for the detection of the less abundant oligomers. Samples were infused at room temperature. The IMS–MS studies were performed to detect and characterize the conformation of the peptide, and ion mobility data acquisition was performed to detect and characterize the conformational changes of the native state monomer and any oligomers that may have formed during the incubation.

Samples were then diluted to create 220, 2200, and 22,000 pmol/µl solutions to perform a 10-, 100-, and 1000-fold molar excess for the binding experiments with Aβ42.

We reconstituted 1 mg of recombinant human Aβ42 peptide in 200 µl of Fisher Optima LC/MS grade water and vortexed vigorously to solubilize to a 5 mg/ml solution. Samples were then diluted to their final concentrations prior to incubation. The sample mixtures were incubated at room temperature for 0, 4, and 24 h, followed by analysis as described in the previous subsections.

3.3 Nuclear Magnetic Resonance Spectroscopy

3.3.1 Aβ42 Preparation

15N-uniformly labeled Aβ42 peptide was purchased from rPeptide (Bogart, GA, USA) and used without further purification. The buffer system described by Roche et al. [15], except for NaOH, was used to acquire the NMR data of Aβ42 titrated with tramiprosate (90% H2O/10% D2O sodium phosphate buffer, pH 7.4 at 37 °C). NaOH was omitted from the sample preparation as it may interfere with tramiprosate binding. The total concentration of Aβ42 in the sample was 75 µM to limit any initial aggregation. The D2O was used to lock the NMR spectrometer.

3.3.2 NMR Experiments

NMR experiments were conducted at 800 MHz on a Bruker AVANCE II spectrometer using a 5 mm HCN cryogenic probe. The probe sample temperature was initially set to 10 °C then slowly warmed to 25 °C and to 37 °C upon insertion of the sample. Spectra were recorded at both 25 and 37 °C. A 1D 3919 Watergate [16] experiment was first conducted to optimize the water suppression and 1H spectral width for the 2D experiments. A relaxation delay of 1.5 s was used with 128 scans. The 1D Watergate experiment was optimized to suppress the largest peak (H2O) in the spectrum. The optimized parameters were then transferred to the 2D experiments. 2D 1H-15N SOFAST-HMQC with 3919 Watergate were used [16, 17]. A total of 128 increments was acquired in t1 (15N) with 96 scans per increment. A J(15N-1H) coupling of 95 Hz was used. All spectra were processed using TopSpin 3.5. Assignments were taken from the literature [15, 18, 19].

3.4 Human Plasma and Brain Pharmacokinetic Analyses, Cerebrospinal Fluid (CSF) Aβ42 Levels, and Pharmacokinetic–Pharmacodynamic Translation

Plasma and cerebrospinal fluid (CSF) concentrations of tramiprosate were determined in frozen samples at...
78 weeks of the completed North American phase III study using validated LC-MS/MS methods [lower limit of quantitation (LLQ) = 5 and 2.5 ng/ml in plasma and CSF, respectively]. The steady-state drug level in human brain was projected based on the brain/plasma drug exposure relationship derived from a rodent model, assuming comparable brain penetration and intra-cerebral kinetics of tramiprosate between the two species following oral administration [20, 21]. Pharmacokinetic data analyses were conducted using WinNonlin Professional v5.0.1 (Pharsight, Mountain View, CA, USA). The CSF Aβ42 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) in patients with AD in the tramiprosate phase II trial as previously described [22] and were used in the present pharmacokinetic–pharmacodynamic analyses.

4 Results

4.1 Multi-Ligand Binding Mode of Tramiprosate and Effects on Aβ42 Monomer Conformation

To address the high conformational flexibility of Aβ42 and characterize its interaction with tramiprosate, we used IMS with a Q-TOF MS with traveling wave ion mobility. IMS is a powerful technique capable of separating molecular ions based on their size and conformation and can also be used to characterize the stoichiometry of ligand–protein complexes [23].

This IMS–MS analysis (Fig. 3) illustrated both the stoichiometry of the drug–protein complex and the shape of Aβ42 and also showed that multiple molecules of tramiprosate bind to a single molecule of Aβ42, in agreement with the previous studies [24]. These results indicate that tramiprosate formed a dynamic solvation envelope surrounding Aβ42 that interacted with the peptide in a dynamic manner. Figure 3 also shows that Aβ42 alone adopted many different conformations, as indicated by a long yellow zone and how the multitude of those conformations changed with each additional bound molecule of tramiprosate. Analysis of the arrival time distribution clearly showed this conformational shift. As additional tramiprosate molecules interacted with Aβ42 monomer, many conformations of Aβ42 transitioned into a more compact, presumably semi-cyclic, conformation (Sect. 4.4). The most extended conformations of Aβ42 on the right part of the yellow zone gradually disappeared with each additional bound molecule of tramiprosate, indicating the formation of more compact and stabilized conformations. With three or more bound tramiprosate molecules, only the most compact conformer populations, and none of the extended populations, were detected. This suggests that the binding of the drug to the peptide has a significant effect on the generation of more defined and stabilized populations of Aβ42 conformers. Additional bound molecules of the drug further stabilized the narrow peak of the population of Aβ42 conformers.

4.2 Tramiprosate Prevents Formation of Aβ42 Oligomers

We next evaluated whether the Aβ42 conformation-stabilizing activity of tramiprosate affects aggregation,
specifically the oligomer aggregation stages from monomers through soluble decamer species. To this end, we examined the formation of soluble Aβ42 oligomers in the absence or presence of tramiprosate by IMS–MS (Figs. 4, 5; Table 1). As expected, the critical neurotoxic oligomers (i.e. dimer, trimer, tetramer, pentamer, hexamer, and decamer) [25, 26] formed following an incubation of Aβ42 monomers; the identities of the oligomer species were further characterized at multiple charge states. However, in the presence of 1000-fold molar excess tramiprosate, the formation of the corresponding oligomers was inhibited. To explore a concentration–response relationship, we incubated monomeric Aβ42 with a 100-fold or 1000-fold molar excess of tramiprosate for 24 h. At a 100-fold molar excess, tramiprosate partially reduced the number of detectable oligomers. Strikingly, at 1000-fold molar excess, tramiprosate completely abrogated the full range of Aβ42 oligomer species (Table 2). Together, the results showed a concentration-dependent effect for tramiprosate in preventing the formation of Aβ42 oligomers, with complete inhibition achieved at the highest concentration tested (i.e., 1000-fold molar excess). Importantly, these findings suggest that tramiprosate stabilizes Aβ42 in its monomeric form and prevents the initiation stage of Aβ42 aggregation.

Together, these data show that the tramiprosate-enveloping mechanism, wherein Aβ42 peptide is enveloped by a cloud of tramiprosate reminiscent of a solvation effect (Sect. 4.4), has implications for clinical activity, especially because high molar excess of the tramiprosate was required in the clinical trials [11].

4.3 NMR Experiments Identify Aβ42 Residues that Interact with Tramiprosate

Next, we used 2D heteronuclear multiple quantum correlation NMR spectroscopy (2D 1H-15N HMQC NMR) of uniformly 15N-labeled Aβ42 peptide (in 90% H2O/10% D2O sodium phosphate buffer, pH 7.4 at 37 °C) to determine how tramiprosate binds to the peptide. Based on the peak dispersion of the spectrum (Fig. 6), monomeric Aβ42 adopted a random conformation, as expected [15]. The 2D 1H-15N HMQC NMR experiments were conducted on samples containing 75 μM Aβ42 titrated with tramiprosate to produce tramiprosate to Aβ42 ratios of 10:1, 100:1, 500:1, 1000:1, 3000:1, and 5000:1. Peak assignments of Aβ42 titrated with tramiprosate were then compared with 2D 1H-15N HMQC spectra of Aβ42 alone. When a 1000-fold excess of tramiprosate was added to the peptide solution, significant chemical shift perturbations were observed. No change was observed in the Aβ42 1H-15N HMQC spectrum at a ratio of 10:1 tramiprosate to Aβ42, but minor changes were observed at a ratio of 100:1. Significant changes in the 2D 1H-15N HMQC peaks began to arise at the ratio of 500:1, which plateaued (i.e., reached a steady state) at a ratio of 1000:1 tramiprosate to Aβ42. Further increases of the ratio to 3000:1 and 5000:1 had no effect on the chemical shift perturbation.

At a 1000-fold excess of tramiprosate over Aβ42, 22 Aβ42 residues showed significant chemical shift perturbations. The most dramatic changes were observed for R5, H6, S8, G9, Y10, K16, L17, V18, F19, N27, K28, and M35. The 2D 1H-15N HMQC peaks from these residues
exhibited at least a 10 Hz chemical shift change in the $^1$H dimension, with K16 and K28 having chemical shift perturbations of 13.5 and 16.1 Hz, respectively, indicating a substantial interaction with tramiprosate. E3, V12, H13, H14, D23, S26, G25, G33, V36, and V39 showed smaller, yet still significant, chemical shift perturbations, indicating that they also interact with tramiprosate. Taken together, these results show that tramiprosate interacts with residues that span the length of Aβ42 in a concentration-dependent mode, which supports the IMS–MS data. Importantly, the strong tramiprosate binding to K16 and K28 supports tramiprosate-mediated disruption of the Lys28-Asp23 and/or Lys28-Glu22 salt bridges and suppression of neurotoxicity and misfolding [7, 27–29], given that these two lysine residues have been previously demonstrated to play a key role in mediation of these activities [8].

4.4 Molecular and Conformational Dynamics

Given the intrinsically disordered nature of Aβ42 and a high conformational dynamics, the interaction with tramiprosate is unlikely to be described by a static structural model with a single tramiprosate molecule bound. Hence, commonly applied structure-based drug-discovery approaches such as molecular docking are unlikely to provide a complete understanding of the MOA of tramiprosate. This represents a challenge to the characterization of the secondary structures of Aβ42 peptides because of their disordered nature and high aggregation propensity.

The characterization of free energy landscapes has been successful in rationalizing the conformational and folding behavior of such disordered proteins, and it provides a concrete representation of the conformational states of such proteins. A previous study [30] described changes in conformation from an α-helical structure to a disordered state, with portions of the peptide adopting a β-sheet structure described by a molecular dynamics simulation. To characterize the structure of Aβ42 alone and with different levels of excess tramiprosate, we performed a series of all atom molecular dynamics simulations. In the absence of tramiprosate, in water alone, Aβ42 adopted a number of very different conformations and was characterized by a disordered structure (Fig. 7a), which is in agreement with other published findings [30]. However, in the presence of increasing concentrations of tramiprosate, the peptide assumed a more conformationally stable form. The observed increase in conformational stability was concentration dependent. A two orders of magnitude molar excess
of tramiprosate forced the protein to adopt a semi-cyclic conformation that was stabilized further by a salt bridge formed by the Asp1 N-terminal amino group and the C-terminal carboxylate of Ala42. This semi-cyclic conformation remained stable in the presence of tramiprosate. The Aβ42 was enveloped by multiple molecules of the drug, which interacted with many transient binding sites in a very dynamic manner. Figure 7b shows a molecular dynamics screenshot with six molecules of tramiprosate binding to a semi-cyclic Aβ42 conformer.

To describe the large conformational changes observed in these simulations, we performed a PC analysis of the free energy surface. This analysis distills the complex motions of a flexible protein into the largest uncorrelated motions, or PCs. The first major motion (PC1) of Aβ42 can be described as a bending of the two helices towards each other like a hinge, and the second motion (PC2) can be described as a twisting of the two helices. Without tramiprosate, Aβ42 exhibited a typical trait of intrinsically disordered proteins: it lacked a narrow, well-defined energy minimum for any single folded structure (Fig. 7c). When PC1 and PC2 were mapped according to their free energy, a number of energy wells were observed (Fig. 7c), which correspond to the multiple Aβ42 conformations detected experimentally via IMS–MS. The 1% tramiprosate solution, corresponding to an Aβ42:tramiprosate molar ratio of 1:250, stabilized the peptide in the semi-cyclic conformation; the energy surface as described by PC analysis showed stabilization of the semi-cyclic conformation as a well-defined energy well (Fig. 7d). This correlates well with the conformer stabilization detected by IMS–MS arrival time distribution (Fig. 3). The stabilization of a single conformation prevents Aβ42 from changing form and aggregating into pathogenic oligomers. Both in the stabilization of a single conformation and in the

### Table 1 Detection of amyloid beta Aβ42 oligomers by ion mobility spectrometry–mass spectrometry in the absence and presence of tramiprosate

| Oligomer | Charge state | m/z (average mass) |
|----------|--------------|--------------------|
| Monomer  | +3           | 1505.72            |
| Monomer  | +4           | 1129.57            |
| Monomer  | +5           | 903.75             |
| Dimer    | +7           | 1290.63            |
| Trimer   | +7           | 1935.62            |
| Trimer   | +13          | 1060.45            |
| Trimer   | +15          | 893.55             |
| Trimer   | +16          | 848.55             |
| Trimer   | +17          | 796.76             |
| Tetramer | +10          | 1806.43            |
| Tetramer | +15          | 1211.74            |
| Tetramer | +18          | 1130.33            |
| Pentamer | +10          | 2257.57            |
| Pentamer | +14          | 1597.45            |
| Pentamer | +16          | 1413.57            |
| Pentamer | +20          | 1131.15            |
| Hexamer  | +21          | 1293.15            |
| Decamer  | +20          | 2257.75            |

Detection of Aβ42 oligomers in the absence of tramiprosate

Detection of monomers only in the presence of tramiprosate

| Oligomer | Charge state | m/z (average mass) |
|----------|--------------|--------------------|
| Monomer  | +3           | 1505.72            |
| Monomer  | +4           | 1129.57            |
| Monomer  | +5           | 903.75             |

### Table 2 Detection of amyloid beta Aβ42 oligomers by ion mobility spectrometry–mass spectrometry in the absence vs. presence of tramiprosate

| Aβ42 oligomer | Aβ42, no tramiprosate | Aβ42:TR 1:100 | Aβ42:TR 1:1000 |
|---------------|-----------------------|---------------|----------------|
| Monomer       | Yes                   | Yes           | Yes            |
| Dimer         | Yes                   | Yes           | No             |
| Trimer        | Yes                   | Yes           | No             |
| Tetramer      | Yes                   | Yes           | No             |
| Pentamer      | Yes                   | Yes           | No             |
| Hexamer       | Yes                   | Yes           | No             |
| Decamer       | Yes                   | No            | No             |

Aβ amyloid beta, TR tramiprosate

Monomeric Aβ42 was incubated in the absence or presence of tramiprosate for 24 h and then analyzed using ion mobility spectrometry–mass spectrometry. ‘Yes’ indicates oligomeric species were detected; ‘no’ indicates absence

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502 P. Kocis et al.
characterization of multiple transient tramiprosate binding sites, these results correlate with the IMS–MS experiments, where we detected up to 13 molecules of tramiprosate bound to A\(_\text{β}42\), in agreement with previous MS data [24, 31]. Interestingly, tramiprosate above 3 mM concentrations did not bind to plasma proteins from human, dogs, and rats in a standard plasma protein-binding study using an ultrafiltration technique [32], suggesting an absence of non-specific binding to plasma proteins such as albumin (data not shown).

Molecular dynamics simulations with free energy landscape analysis predicted a strong effect of tramiprosate on the intrinsically disordered conformations of A\(_\text{β}42\); the effect leads to a defined population of semi-cyclic conformers characterized as a stabilized energy well in the PC plot (Fig. 7d). Structurally, this conformation has a cyclic nature and retains more order than A\(_\text{β}42\) alone. Visual inspection of the simulations demonstrated the transient binding and unbinding of numerous tramiprosate molecules simultaneously along the A\(_\text{β}42\) surface. All of these interactions form a dynamic equilibrium, leading to a very tight conformer population. Taken together, these results suggest that tramiprosate stabilizes the semi-cyclic conformation of A\(_\text{β}42\) and prevents the formation of an initiation seed, thus preventing aggregation of the peptide (Table 3).

4.5 Translational Analyses of Human Brain Drug Exposure vs. the Target

Tramiprosate was measured in the CSF specimens of patients with AD at week 78 from the phase III study [10, 22, 33], and its average concentration at the top tramiprosate dose of 150 mg twice a day (bid) was 60.4 nM (n = 11). Furthermore, based on the brain tissue/plasma exposure ratio derived from rodents and human plasma drug exposure at week 78 after tramiprosate 150 mg bid, we projected the steady-state tramiprosate concentration in brain parenchyma to be approximately 130 nM (Table 4).

In an earlier phase II trial in patients with AD, tramiprosate produced a dose-related CSF A\(_{\text{β}}42\) reduction, suggesting target engagement [22]. In this study, the basal mean CSF concentration of A\(_{\text{β}}42\) was 179 ± 101 pg/ml (i.e., 0.04 nM, n = 46; Table 5); this concentration aligns with the reported CSF A\(_{\text{β}}42\) levels that ranged from 144 to 500 pg/ml [4, 34–36] corresponding to 0.035–0.1 nM, in patients with AD (n = 100) [34] or prodromal/early-stage AD (n = 100) [35, 36], as measured by ELISA or MS. Furthermore, brain A\(_{\text{β}}42\) measures vary in the AD literature, but the reported microdialysis studies in humans have shown that brain interstitial soluble A\(_{\text{β}}42\) are approximately equivalent to CSF A\(_{\text{β}}42\) levels.
[37, 38], and, therefore, the latter can be used as a suitable surrogate for brain pharmacokinetic–pharmacodynamic analyses. Thus, when comparing the ratio of brain tramiprosate : Aβ42, there is an approximately 1300- to 3700-fold excess of tramiprosate over soluble Aβ42 at the steady state based on tramiprosate measured in the brain from patients with AD (Table 4), sufficient to exert a full therapeutic effect of tramiprosate. This analysis fully aligns with the molecular stoichiometry as characterized by the IMS–MS, NMR, and molecular dynamics approaches.

5 Discussion

In this study, we identified a novel enveloping MOA for the small-molecule Aβ-anti-aggregation agent tramiprosate. This mechanism is characterized by a multi-ligand stoichiometry, a critical excess of the ligand to target ratio, and a dose-dependent modulation of the Aβ42 conformational space, resulting in a more stabilized semi-cyclic conformation of Aβ42 and, eventually, the prevention of neurotoxic Aβ42 oligomer formation. This MOA may be responsible for the clinical cognitive and functional

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benefits of tramiprosate as previously observed in patients with mild-to-moderate AD [11].

Specifically, at the molecular level, we showed that tramiprosate enveloped soluble Aβ42 monomers and prevented their self-assembly into the primary monomeric misfolded Aβ42 conformation, and consequently arrested the initiation phase of Aβ42 aggregation, thus preventing the formation of neurotoxic Aβ42 oligomer species. This enveloping mechanism exerted a surprising and significant degree of control over the Aβ42 conformational landscape. This finding is important, especially considering that the tramiprosate molecule is very small (139 Da) yet capable of controlling the structural flexibility of a large peptide/small protein such as Aβ42 under the determined conditions. This may also provide insights for a better understanding of the protein–protein and protein–peptide interactions.

### Table 3  Summary of the ion mobility spectrometry–mass spectrometry, nuclear magnetic resonance, and molecular dynamics data

| IMS–MS | NMR | MD |
|--------|-----|----|
| Aβ42 Oligomer | Aβ:TR | Aβ:TR | Aβ:TR |
| | 1:100 | 1:1000 | 1:1000 |
| Monomer | Yes | Yes | No |
| Dimer | Yes | Yes | No |
| Trimer | Yes | Yes | No |
| Tetramer | Yes | Yes | No |
| Pentamer | Yes | No | No |
| Hexamer | Yes | Yes | No |
| Decamer | Yes | No | No |
| Dose-dependent inhibition of oligomer formation | Dose-dependent chemical shift perturbations | Dose-dependent effect on conformation |

The Aβ42:tramiprosate ratio-dependent effect was consistent across all three techniques. A complete prevention of Aβ42 oligomers was achieved at 1:1000 molar ratio, which is the ratio reaching a maximum interaction of tramiprosate with Aβ42 detected by NMR, and is also predicted by MD with suggested semi-cyclic conformation.

Aβ amyloid beta, Aβ:TR amyloid beta to tramiprosate ratio, IMS–MS ion mobility spectrometry–mass spectrometry, NMR nuclear magnetic resonance, MD molecular dynamics, TR tramiprosate

### Table 4  Steady-state plasma, cerebrospinal fluid, and brain drug exposures following oral administration of tramiprosate 150 mg twice daily in the phase III study

| Parameters | Drug exposure |
|------------|---------------|
| Plasma mean tramiprosate AUC₀–₁₂h | 4429 ng/ml × h (31.8 μM × h) |
| CSF mean tramiprosate concentration (Cₜᵣ₋₅₀) | 60.4 nM |
| Projected brain mean tramiprosate concentration (Cₜᵣ₋₅₀) | 130 nM |
| CSF Aβ42 concentration | 0.04 nM (tramiprosate phase II study [34] 0.035–0.1 nM [35–37]) |
| Brain soluble Aβ42 concentration | Comparable to CSF based on published human microdialysis studies [37, 38] |
| Multiple excess of brain drug vs. soluble Aβ42 | 1300- to 3700-fold |

Aβ amyloid beta, AUC area under curve, bid twice daily, CSF cerebrospinal fluid

* Estimated based on the brain/plasma AUC exposure relationship obtained from the rodents and an accumulation factor of 1.3 in the brain at steady state

### Table 5  Concentration of amyloid beta Aβ42 in the cerebrospinal fluid of subjects with mild to moderate Alzheimer’s disease (n = 46) [11]

| Age of subjects with AD in trial | Baseline MMSE | CSF Aβ42 (pg/ml) |
|----------------------------------|---------------|------------------|
| 75.1 ± 8.3 years | 19.4 ± 0.8 | 179 ± 101 |

Data are mean ± SD

AD Alzheimer’s disease, CSF cerebrospinal fluid, MMSE Mini Mental State Examination
interaction processes in a living organism and in disease states. The challenge to modulate Aβ42 conformational dynamics has been one of the major reasons that this relatively small protein has been such an elusive target in AD drug development. We hypothesize that the enveloping occurs after a critical mass of tramiprosate (i.e. a sufficient concentration relative to Aβ42 monomer) is reached in the CNS. Because of the relatively weak nature of the transient binding of tramiprosate to Aβ42, the monomeric peptide requires a large excess of tramiprosate molecules to overcome the rapid off rates. Thus, the binding and unbinding occur rapidly enough that, only at a ratio of approximately 1:1000 of Aβ42:tramiprosate (at the ratio of 1:500, functional interaction becomes measurable), Aβ42 becomes enveloped by the drug and a full inhibition of oligomer formation is achieved.

Our molecular dynamics calculations showed a multiligand interaction of tramiprosate with both anions and cations of the Aβ42 side chains. Our NMR study identified the interaction of tramiprosate’s sulfonic anion with Lys16 and Lys28 as the strongest. Important roles for both amino groups have been established previously [39]. Relevant to the former, a recent study [8] showed that replacement of Lys16 with Ala abolished or dramatically reduced the neurotoxicity of Aβ40 and Aβ42, suggesting that tramiprosate binding to Lys16 may have a similar effect. The interaction of tramiprosate with Lys28 is particularly critical, because it potentially disrupts the formation of Lys28–Asp23 salt bridges (Fig. 8). Several groups [8, 40–44] have shown that the Lys28–Asp23 intramolecular salt bridge stabilizes the conformation that is important for seed formation and Lys28–Asp23 intermolecular salt bridges that form the basis of amyloid relays [45] (Fig. 8).

The potential impact of tramiprosate is that its anti-aggregation MOA is upstream of the Aβ oligomer formation cascade. Based on principles of biomolecular recognition and the present results, it is reasonable to suggest that, because of its interaction with Lys28, tramiprosate may prevent and block the formation of all salt bridges that are key for the salt bridge between Lys28 and Ala42, Glu22 or Asp23. This may have implications for tramiprosate and its MOA in light of recent structural characterizations of amyloid aggregates with Lys28–Ala42 salt bridges [42, 43].

Another important consideration is the putative endogenous role of Aβ42 monomers in brain. To date, the physiological role of Aβ42 is not fully understood. For another aggregating protein, α-synuclein, which is implicated in Parkinson’s disease, a simple reduction of its levels is associated with synaptic failure [46], whereas whether a substantive reduction of monomeric Aβ42 levels might also result in detrimental clinical defects is unclear. Thus, therapeutic agents such as tramiprosate that preferentially prevent the formation of oligomers by an upstream action directly on Aβ42 monomers, without affecting Aβ production, unlike beta-secretase 1 (BACE1) inhibitors or γ-secretase inhibitors, may yield a new class of AD therapeutics with improved safety and efficacy.
this MOA, long-term treatment with tramiprosate (over 78 weeks) was well tolerated and devoid of vasogenic edema side effects, also referred to as ARIA (amyloid-related imaging abnormalities reported for some of the immunological therapies), in over 2000 patients with AD treated to date [11].

We also correlated the molecular mechanism results with the clinical pharmacokinetic and efficacy data [10, 11, 22, 33]. The data from our IMS–MS, NMR, and molecular dynamics experiments suggest the requirement of three orders of magnitude excess of tramiprosate relative to soluble Aβ42 to achieve a complete prevention of Aβ42 oligomer formation and aggregation. This excess ratio is in line with the projected tramiprosate concentrations in the CNS in humans based on the present translational pharmacokinetic dose-exposure analyses. The measured steady-state average concentration of tramiprosate in the brain at the dose of 150 mg tramiprosate bid from the phase III North American AD trial was 130 nM, which is 1300- to 3700-fold in excess of human CNS soluble Aβ42 levels based on the data from subjects with AD in the previous tramiprosate clinical trials, as well as the reported range in patients with AD [4, 34, 36]. Importantly, clinical cognitive and functional improvements have been demonstrated in subjects with AD in the tramiprosate phase III AD trial [11]. This suggests that the results from our current mechanism study reflect the therapeutic effect of tramiprosate in patients with AD.

While clinical efficacy of tramiprosate is suggested in a genetically defined subset of patients with AD with high amyloid burden, and its presented mechanistic understanding represents therapeutic promise, it is clear that a single-target approach to AD has not yet yielded an effective therapy. Considering the rather complex pathophysiological features of this disease, which involves multiple molecular,

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**Fig. 9** Solid-state nuclear magnetic resonance (NMR) of amyloid fibril where tetramer is depicted with each individual amyloid beta Aβ42 molecule colored differently to highlight the crosslinking intermolecular salt bridge Lys28–Asp23 (source PDB 2BEG). Thus, red Lys28 forms a salt bridge with yellow Asp23. This intermolecular salt bridge stabilizes the growing superstructure.

**Fig. 10** Recently published high atomic resolution of full molecular structures of amyloid beta Aβ42 aggregates [42, 43] illustrating the salt bridge between Lys28 and C-terminal Ala42 (highlighted and annotated). The figure highlights Lys28–Ala42 as examples of salt bridges in the structures that are to be disrupted by tramiprosate binding.
biochemical, and cellular pathways and systems (e.g., cholinergic function, amyloid, tau, and inflammatory components), combination therapies targeting multiple steps of amyloid cascade (e.g., tramiprosate in combination with BACE1 inhibitors, monoclonal antibodies, or insulin-degrading enzymes, etc.) or both amyloid and non-amyloid pathways (e.g., tramiprosate in combination with tau inhibitors or symptomatic agents), it is likely that future therapies will involve an approach similar to that of precision medicine, which will likely comprise the combination of more than one therapeutic modality tailored to a particular stage of the disease and/or disease phenotype. Important to this point, the clinical efficacy of tramiprosate observed in the phase III North American trial [11] was identified on top of concurrent acetylcholinesterase inhibitors (e.g., donepezil) and memantine and thus represents the first-step combination therapy approach.

6 Conclusion

Our study shows that (1) tramiprosate modulates the Aβ42 conformational landscape in a concentration-dependent manner, resulting in the stabilization of Aβ42 monomers and inhibits the formation of oligomers and subsequent aggregation and (2) the observed molecular stoichiometry is consistent with the clinical drug dose exposure versus target relationship that has been shown to achieve a robust clinically meaningful efficacy in patients with APOE4/4 homozygous AD in the previous phase III trials, suggesting that the MOA findings of tramiprosate most likely underpin its clinical outcome. The discovery of the unique enveloping MOA of tramiprosate may broaden our understanding of the control of conformationally flexible peptides/proteins, which may find potential utility in the development of disease-modifying therapies for AD and related neurodegenerative disorders caused by misfolded proteins.

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Author Contributions PK conceived the enveloping MOA and designed all studies, interpreted the data in collaboration with JH, and wrote the manuscript in collaboration with JH, JY, WS, SR. Molecular dynamics calculations were performed by WS and SR. Pharmacokinetic/pharmacodynamic analyses were performed by JY and JH. All co-authors reviewed and contributed to the manuscript.

Compliance with Ethical Standards

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Conflict of interest PK, MT, JH, and JY are employees of Alzheon Inc. WS and SR are employees of Schrödinger, which had a scientific services agreement in place to perform some of the work in this manuscript. KB has served as a consultant or on advisory boards for Alzheon, Eli Lilly, Fujirebio Europe, IBL, International, Novartis, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. HF has no competing interests.

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Mechanism of Action of Tramiprosate in Alzheimer’s Disease

509

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