Brain Permeable Tafamidis Amide Analogs for Stabilizing TTR and Reducing APP Cleavage

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ABSTRACT: Tafamidis, **1**, a potent transthyretin kinetic stabilizer, weakly inhibits the γ -secretase enzyme *in vitro*. We have synthesized four amide derivatives of **1**. These compounds reduce production of the A β peptide in N2a695 cells but do not inhibit the γ -secretase enzyme in cell-free assays. By performing fluorescence correlation spectroscopy, we have shown that TTR inhibits A β oligomerization and that addition of tafamidis or its amide derivative does not affect TTR's ability to inhibit A β oligomerization. The piperazine amide derivative of tafamidis (**1a**) efficiently penetrates and accumulates in mouse brain and undergoes proteolysis under physiological conditions in mice to produce tafamidis.

KEYWORDS: Alzheimer's disease, A β peptide, γ -secretase, prodrug, tafamidis, transthyretin

Transthyretin (TTR) is a tetrameric carrier protein, transporting thyroid hormone and holo retinol-binding protein.¹ It is secreted by liver into the blood at a steady state concentration of about 3–6 μ M and by the choroid plexus (CP) into the cerebrospinal fluid (CSF) at a steady state concentration of $\approx$ 300 nM.² In brain, TTR counteracts the neurotoxic effects of A β peptides by reducing their aggregation and enhancing clearance of the oligomers and plaques from brain.^{3–5} TTR may also degrade A β peptides.⁶ Neurotoxic A β species seem to initiate Tau-hyperphosphorylation (pTau) and tangle formation that together mediate neuronal damage and memory loss, leading to Alzheimer's disease (AD).⁷ There are multiple studies supporting the protective effects of TTR.⁸ For example, overexpression of the wild-type (WT) human TTR transgene was beneficial, while silencing the endogenous TTR gene or neutralizing TTR protein function by using an anti-TTR antibody accelerated A β aggregation in AD mice.^{4,9}

TTR itself is an amyloidogenic protein. Tetramer dissociation is rate-limiting for TTR aggregation, a process also requiring partial monomer denaturation.^{10,11} There is very strong genetic and pharmacologic evidence that TTR aggregation causes the degenerative phenotypes in the TTR amyloidoses.^{12–14} The TTR amyloidoses can present as a cardiomyopathy or a peripheral neuropathy,¹⁵ whereas some mutations lead to neurodegeneration of the central nervous system or the eye initially.¹⁶ If untreated, multiple organ systems become affected upon progression. There is persuasive evidence that pharmacologic kinetic stabilization of the native tetrameric quaternary structure of TTR slows or stops the progression of the TTR amyloidoses.^{13,17} A small molecule kinetic stabilizer could potentially benefit AD patients provided that it does not adversely affect the TTR–A β interaction.

Indeed, it has been shown that the TTR effect on A β clearance increases when TTR is pharmacologically stabilized.¹⁸ There are conflicting reports though that TTR stabilization via binding at the T4-site affects the TTR–A β interaction adversely.^{8,19}

Herein, we explore the benefits of using the Food and Drug Administration (FDA) approved TTR kinetic stabilizer tafamidis (**1**, Figure 1)¹⁷ for lowering A β oligomerization and production in the Alzheimer's disease context.²⁰ Toward this end, we have prepared amide derivatives of **1** as potential inhibitors of A β oligomerization and production. We have found that tafamidis weakly inhibits γ -secretase activity in a cell-free assay, whereas several amide derivatives of **1** and analogs thereof reduce A β production in cells independent of γ -secretase inhibition. Notably, compound **1** and its amide derivatives do not compromise TTR's ability to reduce A β oligomerization. We have also shown that compound **1a**, the piperazine amide of **1**, accumulates in brain and is partially hydrolyzed to **1**, providing proof-of-principle for a tafamidis prodrug strategy for stabilizing TTR in the brain and in the periphery.

CHEMISTRY

The synthesis of amide derivative **1a** was inspired by the structures of compounds **2**²¹ and **2a**²² (Figure 1), which are

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Figure 1. Comparison of the FDA approved TTR stabilizer tafamidis, **1**, with DV2-103, **2**, and **2a** reveals **1** as a suitable pharmacophore for developing compounds with inhibitory effects on A β production. Shown in blue and/or red color are the fragments in **1** and **1a** identical to some fragments in **2** and **2a**.

kinase inactive analogs of PD173955²³ and reduce production of A β peptides with micromolar EC₅₀, similar to Gleevec and analogs.^{24,25} Compounds **1** and **1a** have some substructures in common with **2** and **2a**, Figure 1. We prepared compound **1a** by forming an amide bond between acid **1**²⁶ and the Boc-protected amine **a**, followed by Boc-deprotection using 4 M HCl in dioxane (Figure 2A, upper row). We similarly synthesized additional amides of **1** (Figure 2A, upper row), as well as amide analogs **3–9** (Figure 2B), wherein substituents on the core tafamidis structure were varied.

SCREENING AND EVALUATION

We performed screening of **1** and the amide derivatives **1a–i**, **3a–b**, **3e–g**, **4b**, **5a–b**, **6a**, **7a**, **8a**, and **9a** (Figure 2) and

determined their effects on A β production in N2a695 cells. The latter express human WT amyloid precursor protein 695 (APP695), which undergoes two successive cleavages to produce human A β peptides.^{27,28} First β -secretase or β -cleavage shreds the large extracellular portion of APP as a soluble APP fragment (sAPP β) leaving the membrane-bound C-terminal fragment (β CTF or C99). Subsequent γ -secretase or γ -cleavage of β CTF leads to multiple A β isoforms and the APP intracellular domain (AICD). For the A β production assay, we incubated N2a695 cells with candidate compounds (10 μ M) for 6 h at 37 °C and collected the culture media and cell lysates, as described.^{22,25} We quantified the levels of A β 40 and A β 42, which are the most prominent and relevant A β isoforms in AD, in culture media using commercially available human A β ELISA kits. We found that four compounds, **1a**, **1h**, **3a**, and **3e**, reduced production of both A β 40 and A β 42 peptides by 30–60% compared to the DMSO (vehicle) control (See: SI Figure S-1 and Figure 3A). To confirm that the effect of compounds **1a**, **1h**, **3a**, and **3e** on A β 40 and A β 42 production is not due to any toxicity, we incubated N2a cells with each compound (10 μ M) in a 96-well plate for 24 h and determined the number of viable cells using MTT assay.²⁹ Although, we observed some cellular toxicity with compounds **1h** and **3a** after 24 h of treatment (SI Figure S-2), it should not have affected the A β production, because cells were incubated with the compounds for only 6 h in the A β production assay.^{22,25}

Subsequently, we determined the effects of **1**, **1a**, **1h**, **3a**, and **3e** on APP cleavage in comparison to DMSO controls, in N2a695 cells by performing Western blotting (WB) of cell lysates. We probed SDS-PAGE gels with antibody RU369, which recognizes the C-terminus of APP fragments. The results, shown in Figure 3B, clearly revealed an increase in APP-CTF in samples treated with compounds **1a**, **1h**, **3a**, and **3e**, but not in the vehicle control (DMSO) or upon treatment

Figure 2. Structure and synthesis of various amide derivatives of tafamidis (A) and of the analogous acids (B). Note: Step “i” was used with all compounds, and step “ii” for the Boc-protected intermediates that were obtained in step “i” using the Boc-protected amines **a'**, **c'**, or **h'**.

Figure 3. Tafamidis derivatives **1a** and **1h** and analogous compounds **3a** and **3e** reduce Aβ production in N2a695 cells by affecting APP cleavages. Bar graphs are the measure of Aβ40 and Aβ42 peptides in cell supernatants post treatment with these compounds and determined using human Aβ ELISA kits (A), Western blots (lower) and quantitation of the APP-CTF levels (upper) of N2a695 cell lysates post compound treatment (B), and effect of compound **1** on γ-secretase activity measured *in vitro* (C). All experiments were performed at least twice in duplicate or triplicate, except for compound **3a**, for which Aβ42 shown in part A was measured only once. Data shown in part C are from one experiment in duplicate. Statistical significance (P): *, <0.05%; **, <0.01%; ***, <0.001%.

Figure 4. TTR inhibits Aβ polymerization in both the presence or absence of **1** and **1a**, as determined using FCS. Shown are fitted autocorrelation curves for diffusion of decreasing Aβ (Aβ40:Aβ42, 1:1) particle concentrations as a function of time. (A) The amplitude of the autocorrelation curve at a given diffusion time (τ = 10⁻⁴ s, red dashed-line arrow) increases along the reaction time point 0, 2, and 4 h representing the decreasing particle numbers as a result of Aβ polymerization. In addition, the autocorrelation functions show increasing contribution from long delay times, indicating the increasing size of Aβ particles. A minimum change in autocorrelation curves was found when Aβ polymerization was followed in the presence of: (B) TTR, (C) TTR + **1**, or (D) TTR + **1a**, over a 4-h period, indicating that TTR inhibited Aβ polymerization in both the presence or absence of **1** and **1a**. Lower autocorrelation curves found with TTR + **1a** indicate a strong beneficial effect of compound **1a** on TTR-mediated Aβ aggregation inhibition.

with **1**. These results suggest γ-secretase inhibition or α-secretase activation. The α-secretases are a group of metalloenzymes known to mediate nonamyloidogenic cleavage of APP. However, by performing γ-secretase activity assay, *in*

vitro,³⁰ we found that compounds **1a**, **1h**, **3a**, and **3e** did not inhibit γ-secretase activity appreciably at 30 μM or lower concentration (Figure 3C). In contrast, compound **1**, which did not show activity in N2a695 cells, inhibited γ-secretase in a

dose dependent manner, with an apparent IC_{50} of $<30 \mu M$. We further studied tafamidis amide **1a**. Compound **1a** is nontoxic to cells for extended incubation periods (SI Figure S-2).

Poor clearance of the aggregation-prone $A\beta$ peptide leads to oligomers and plaque build-up in the brain. *In vivo* studies with an anti-TTR antibody in a mouse model have earlier shown that neutralizing the TTR protein increases $A\beta$ load.⁹ Conversely, increasing TTR expression reduces $A\beta$ load, likely through reducing $A\beta$ oligomerization and enhancing $A\beta$ clearance.³¹ To test that compounds **1** and **1a** do not interfere with the $A\beta$ /TTR interaction, we used fluorescence correlation spectroscopy (FCS), which conveniently distinguishes and predicts the levels (particle number) of smaller vs the larger $A\beta$ particles.³² In this study, the $A\beta$ peptide, mixed with a minute amount of a fluorophore-labeled $A\beta$ peptide, is used and temporal fluctuations of fluorescence caused by diffusion of fluorophore-labeled $A\beta$ peptide passing through a small confocal volume (<1 fL) are recorded. Samples containing monomeric $A\beta$ are expected to show lower autocorrelation functions in comparison to oligomeric $A\beta$. To perform FCS, $10 \mu M$ $A\beta$ peptide ($A\beta_{40}$ and $A\beta_{42}$ in 1:1 ratio), containing 2 nM of tetramethylrhodamine (TMR)-labeled $A\beta_{40}$, and 2 nM cholesterol were distributed in a spectroscopy 8-well plate. Fluorescence intensity and frequency of the intensity fluctuations in each well were recorded at 0, 2, and 4-h time point.

It became evident that significant $A\beta$ aggregation occurred over a 4-h period, as indicated by the large amplitude increase in the autocorrelation curve between 2 and 4 h (Figure 4A, red dashed arrow) in the absence of TTR. This did not occur in the presence of TTR (Figure 4B), indicating a strong inhibition effect of TTR on $A\beta$ aggregation.³³ To test whether tafamidis or the amide derivative **1a** might interfere with the ability of TTR to inhibit $A\beta$ aggregation, addition of compound **1** or **1a** to $A\beta$ and TTR results in only fractional changes in diffusion times over 4 h, indicating that under our experimental conditions transition from monomer to aggregated $A\beta$ is minimal (Figure 4C–D). Notably, when $A\beta$ /TTR solution was mixed with **1a** the autocorrelation curves displayed much lower diffusion times in comparison to $A\beta$ /TTR solution alone or upon adding **1** to $A\beta$ and TTR (Figure 4D vs 4B–C). This indicated a slower phase of $A\beta$ aggregation with **1a**, as the concentration of $A\beta$, which could be reflected by the autocorrelation curves in Figure 4D, remains high over a 4 h period. However, in the absence of TTR, compound **1** or **1a** was unable to inhibit $A\beta$ aggregation (SI Figure S-4). In summary, these results suggest that usage of **1** or **1a** has no discernible adverse effect on the beneficial effect of TTR on inhibiting $A\beta$ oligomerization.

Next, we determined brain permeability of compound **1a** in 3-month old 5xFAD mice.³⁴ These mice exhibit $A\beta$ pathology within 3 to 6 months and are suitable for performing this efficacy study, *in vivo*. Mice were dosed with compound **1a** in the drinking water for 2 weeks, and the concentration of **1a** in plasma and in brain tissue was determined using LC-MS/MS.³⁵ Because amide **1a** could undergo proteolysis to afford tafamidis in the stomach, and/or the plasma, and/or in brain, we also determined the concentration of **1** in both blood and brain tissue extracts. As shown in Figure 5A, the piperazine amide derivative of tafamidis (**1a**) appears to penetrate mouse brain (Figure 5A), although the blood brain barrier (BBB) in 5xFAD mice is known to be compromised. In future studies, it is important to rule out blood contamination of brain extracts.

Figure 5. Brain penetration and activity of tafamidis and the amide derivative **1a**. (A) Brain and serum concentration of **1a** and product **1**. Compound **1a** (50 mg/kg) was given to 3 months old 5xFAD mice ($n = 3$) for 15 days. Blood and brain tissues were collected on the 15th day and analyzed to determine the concentration of **1a** and **1** using LC-MS/MS. (B) Microsome stability of **1a**, measured by incubating it with mouse and human liver microsomes and determining the remaining **1a** periodically using LC-MS/MS. (C) Effects of compound **1a** on TTR stabilization as compared to tafamidis, **1**, and determined using TTR subunit exchange assay.

Amide **1a** underwent proteolysis in mice to produce tafamidis, detected both in murine brain and in plasma when mice consumed **1a** in the drinking water over a period of 15 days.

To estimate the pharmacokinetics of compound **1a** in humans, we determined its metabolic stability using human and mouse liver microsomes and determined amounts of the nonmetabolized **1a** using methodology, as described.^{25,36} We incubated **1a** at two concentrations (1 and 10 μM) with mouse and human liver microsomes for 30 and 60 min at 25 °C, before the remaining amounts of **1a** were quantified. The results, tabulated in Figure 5B, established that compound **1a** is likely more stable in humans than in mice. The stability of **1a** in human or murine plasma was not established.

Compound **1a** appears to penetrate the BBB and undergo hydrolysis in the brain and in the plasma to produce tafamidis, providing proof-of-principle that analogous compounds could become compound **1** prodrugs. Since there are both **1** and **1a** in plasma and brain samples, we probed the ability of both compounds to stabilize TTR in plasma using the TTR subunit exchange assay.^{17,37,38} Prior to the initiation of subunit exchange, compound **1** or **1a** was added to healthy donor plasma at concentrations ranging from 0 to 60 μM . Following a 30 min incubation period at 25 °C, subunit exchange assays were initiated by adding 1.8 μM recombinant dual-FLAG-tagged WT (FT₂-WT) TTR to the plasma samples. The results, shown in Figure 5C, clearly demonstrate that compound **1a** stabilizes TTR, but not as well as tafamidis

does. WT TTR subunit exchange, quantified by k_{ex} (subunit exchange rate or the rate of tetramer dissociation), proceeds faster with **1a** than with **1** below a concentration of 30 μM .

In conclusion, amide derivatives of tafamidis and of compound **3** significantly reduced production of A β peptides in N2a695 cells. There was an increase in the levels of CTFs suggesting that the test compounds inhibited the γ -secretase enzyme. By using a sensitive FCS detection method, we provide direct evidence that the inhibitory effects of compound **1** and **1a** on A β oligomerization are TTR dependent. Further investigations are required to characterize the interaction of compound **1a** and TTR on a molecular level. Compound **1a** also provides proof-of-principle that with further refinement, it may be possible to make a brain permeable prodrug of **1**, which has implications for treatment of TTR-associated amyloidosis in the brain and the eye.³⁹

■ EXPERIMENTAL SECTION

Synthesis and Characterization of Compounds. All amide derivatives of acids **1** and **3–9** were prepared by usual HATU coupling of these compounds with various amines, and those possessing N-Boc protection were deprotected using 4 M HCl in dioxane, as described in the SI.

A β Production and APP Metabolism Studies. N2a695 cells (4.0×10^5 – 4.5×10^5 cells/mL, 2 mL/well, 90–95% confluent) were treated with compounds (10 μM final concentration) at 37 °C under 5% CO₂ atmosphere for 6 h. Subsequently, cell media and cell lysates were collected for determining A β levels and APP and CTF levels, respectively. A β concentration was determined by transferring cell culture media (50–100 μL , 5–10 $\times$ dilution for A β 40 and no dilution for A β 42 measurement) to strips of 96-well ELISA plates precoated with the human A β 40 and A β 42 peptide capture antibodies. Further processing was carried out per manufacturer instructions, and signals for A β were measured using PerkinElmer Envision ELISA reader. For determining APP metabolites in cell lysates, the latter (30 μL) were loaded and separated on a 16.5% Tris-Tricine gel (Criterion) and electrotransferred to PVDF membrane (EMD Millipore). The membrane was then treated with glutaraldehyde (Sigma) solution (0.25% in PBS) before it was blocked using milk PBST and probed using antibody RU369, HRP-linked secondary antibody, and enhanced chemiluminescence ECL reagent for detection.

TTR Subunit Exchange Assay. Subunit exchange assays were done as previously described.^{37,38} Briefly, prior to subunit exchange assays, compounds were added *ex vivo* to two healthy donor plasma samples at the indicated concentrations. Samples were then incubated at room temperature for 30 min. Subunit exchange was initiated by the addition of 1.8 μM FT2-WT TTR to 40 μL of the plasma samples which were then incubated at 25 °C for 48 h. At the end of the 48 h incubation, a 10 μL aliquot of the exchange mixture was transferred to a UPLC sample glass vial (Waters) to which 1 μL of 3 mM stock solution of A2, a fluorogenic small molecule (S-(4-fluorophenyl) (E)-3-(dimethylamino)-5-(4-hydroxy-3,5-dimethylstyryl)benzothioate),⁴⁰ was added for a 3 h reaction period, followed by addition of 52 μL of 50 mM phosphate buffer, pH 7.6. Samples were analyzed via ion exchange chromatography and fluorescence from the TTR-A2 conjugate allows detection of TTR tetramers. The rate of subunit exchange was quantified by the rate of appearance of the (WT TTR)₃(FLAG-tagged TTR)₁ heterotetramer-A2 conjugate peak.

FCS Data Acquisition. All FCS measurements were performed on the Zeiss LSM 880 confocal microscope (Carl Zeiss, Thornwood, NY). A 40 $\times$ Water immersion 1.2 NA objective from Carl Zeiss was used to perform all measurements. A 561 nm wavelength laser was used to excite the sample, and the detection range was between 570 and 650 nm. Laser power has been kept very low (between 0.1 and 0.3%) in order to minimize triplet state transitions while keeping the CPM (counts per molecule) rate high. Pinhole alignments were made using free dye in solution at the start of the day of each experiment. For each experiment, 10 traces of 20 s each were recorded.

FCS Data Analysis. Individual autocorrelation curves were calculated using Zeiss Zen Black software (Carl Zeiss, Thornwood, NY) and then plotted in MATLAB (The MathWorks, Natick, MA). The autocorrelation function $G(\tau)$ is defined as

$$G(\tau) = \frac{\langle \delta F(0) \delta F(\tau) \rangle}{\langle F(\tau) \rangle^2} \quad (1)$$

where $F(\tau)$ is the fluorescence obtained from the volume at delay time τ , brackets denote ensemble averages, and $\delta F(\tau) = F(\tau) - \langle F(\tau) \rangle$. The fitting formula is based on the multicomponent diffusion model:

$$G(\tau) = \sum_i \frac{1}{N_i} \left(1 + \frac{4D_i\tau}{w_{xy}^2} \right)^{-1} \left(1 + \frac{4D_i\tau}{w_z^2} \right)^{-1/2} \quad (2)$$

where N is the number of fluorescent particles in the confocal volume, D_i is the diffusion coefficient of the i -th species; w_{xy} and w_z are the effective lateral and axial dimensions of the Gaussian focal volume, respectively, and τ is the lag time. For A β polymerization, we considered the value of i to be 2.⁴¹

Statistical Analysis. The data are presented as means $\pm$ SEM. Data were analyzed by Student's t test for single comparison and one-way ANOVA for multiple comparisons, and those showing P value < 0.05 were considered significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00688>.

General synthetic procedures and characterization of synthesized compounds including ¹H NMR spectra and MS data. Figures showing screening data of all synthetic analogs and evaluation of selected compounds. (PDF)
SMILES data for compounds (XLSX)

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AS, JCC, PX, YHC, WS, YML, PG, JWK, and SCS designed the experiments. AS, JCC, PX, KG, YHC, WS, and XZW performed and analyzed the experiments. AS, JCC, PX, YHC, YML, JWK, and SCS wrote the manuscript.

Notes

The authors declare no competing financial interest.

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