

Aminopyrimidine Class Aggregation Inhibitor Effectively Blocks $\text{A}\beta$ –Fibrinogen Interaction and $\text{A}\beta$ -Induced Contact System Activation

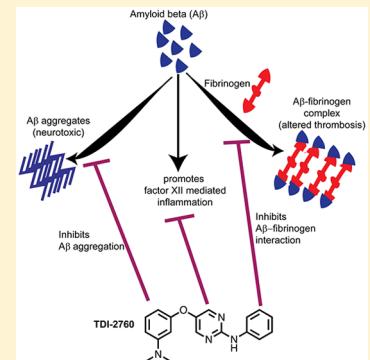
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Supporting Information

ABSTRACT: Accumulating evidence suggests that fibrinogen, a key protein in the coagulation cascade, plays an important role in circulatory dysfunction in Alzheimer's disease (AD). Previous work has shown that the interaction between fibrinogen and β -amyloid ($\text{A}\beta$), a hallmark pathological protein in AD, induces plasmin-resistant abnormal blood clots, delays fibrinolysis, increases inflammation, and aggravates cognitive function in mouse models of AD. Since $\text{A}\beta$ oligomers have a much stronger affinity for fibrinogen than $\text{A}\beta$ monomers, we tested whether amyloid aggregation inhibitors could block the $\text{A}\beta$ –fibrinogen interaction and found that some $\text{A}\beta$ aggregation inhibitors showed moderate inhibitory efficacy against this interaction. We then modified a hit compound so that it not only showed a strong inhibitory efficacy toward the $\text{A}\beta$ –fibrinogen interaction but also retained its potency toward the $\text{A}\beta42$ aggregation inhibition process. Furthermore, our best hit compound, TDI-2760, modulated $\text{A}\beta42$ -induced contact system activation, a pathological condition observed in some AD patients, in addition to inhibiting the $\text{A}\beta$ –fibrinogen interaction and $\text{A}\beta$ aggregation. Thus, TDI-2760 has the potential to lessen vascular abnormalities as well as $\text{A}\beta$ aggregation-driven pathology in AD.



Alzheimer's disease (AD) is a neurodegenerative disorder that leads to profound cognitive decline in the elderly population.^{1,2} AD is a complex, multifactorial disease, and it is likely that various disease mechanisms contribute to pathology.^{3–5} Although the direct neuronal toxicity of the AD-related peptide, β -amyloid ($\text{A}\beta$), is well recognized *in vitro*, the precise mechanism by which $\text{A}\beta$ disrupts neuronal function in AD patients is unclear.^{2,6–8} Increasing evidence suggests that AD may be a neurological disorder in which circulatory deficiencies significantly contribute to the pathophysiology of the disease.^{9–14} Furthermore, it has been shown that $\text{A}\beta$ plays an important role in this cerebrovascular dysfunction in AD.^{15–20} Therefore, some of the neuronal loss observed in AD patients could be secondary to circulatory problems. For example, many AD patients display altered cerebral blood flow, damaged cerebral vasculature, neuroinflammation, and cerebral microinfarcts.^{5,21–24}

Components of the coagulation cascade could contribute to these vascular abnormalities as well as cognitive decline and neurodegeneration in AD.^{17,25} AD patients show increased coagulation factor XII (FXII) activation, kallikrein activity, and high molecular weight kininogen (HK) cleavage in their plasma.¹⁷ Depletion of FXII inhibits HK cleavage as well as reduces neuroinflammation, neurodegeneration, and fibrinogen deposition in the brains of AD mice.²⁵ Moreover, fibrinogen, the end product of the coagulation cascade, interacts with $\text{A}\beta$ with strong affinity, and this interaction increases the

aggregation kinetics of $\text{A}\beta$.²⁶ This interaction also induces abnormal clot structure and increases clot resistance toward fibrinolysis.^{15,18,27,28} In addition, fibrinogen is frequently localized to $\text{A}\beta$ deposits around the blood vessels of the brain (cerebral amyloid angiopathy, CAA) and the brain parenchyma in AD patients as well as AD mouse models.^{15,29–31} These findings suggest that the $\text{A}\beta$ –fibrinogen interaction could be crucial to the onset and progression of neurovascular damage and cognitive impairment in AD. To further support this hypothesis, administration of RU-505, a small compound inhibitor of the $\text{A}\beta$ –fibrinogen interaction, reduced cortical inflammation and vascular $\text{A}\beta$ deposition and lessened the severity of cognitive impairment in AD mouse models.²⁷

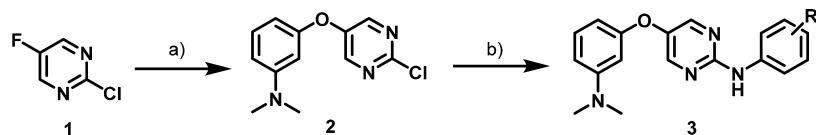
The binding affinity of soluble $\text{A}\beta$ oligomers to fibrinogen is nearly 10-fold higher than that of $\text{A}\beta$ monomers.²⁷ This finding suggests that minimizing the population of $\text{A}\beta$ oligomers could indirectly affect the $\text{A}\beta$ –fibrinogen interaction. In addition, several $\text{A}\beta$ aggregation inhibitors directly bind $\text{A}\beta$,^{32,33} suggesting they may also inhibit the $\text{A}\beta$ –fibrinogen interaction directly. Therefore, we screened several known $\text{A}\beta$ aggregation inhibitors for the ability to block the $\text{A}\beta$ –fibrinogen interaction and found a subset of hits with inhibitor capacity. We then

Received: December 4, 2017

Revised: February 1, 2018

Published: February 2, 2018



Scheme 1. Synthetic Scheme of TDI-2760 Derivatives^a

^aReagents and conditions: (a) K_2CO_3 , DMF, 60 °C; (b) ArNH₂, BrettPhos Pd G3, BrettPhos, *t*-BuOK, DME, 100 °C.

improved the inhibitory efficacy of hit compounds using medicinal chemistry approaches and found that the best hit compound, TDI-2760, not only showed a strong inhibitory efficacy toward the $A\beta$ –fibrinogen interaction but also modulated $A\beta$ 42-induced contact system activation, a pathological condition observed in some AD patients.¹⁷

MATERIALS AND METHODS

Synthesis of $A\beta$ Aggregation Inhibitors. The derivatives of SEN1269 were synthesized as shown in **Scheme 1**.

As described in **Scheme 1**, 2-chloro-5-fluoropyrimidine (**1**) was treated with 3-dimethylaminophenol in the presence of K_2CO_3 to afford chloro analog (**2**). Buchwald amination of **2** using palladium catalyst with various amines worked smoothly to give corresponding amino analogs **3**.

Synthesis of 3-(2-Chloropyrimidin-5-yl)oxy-*N,N*-dimethyl-aniline. A mixture of 3-(dimethylamino) phenol (11.9 g, 87.1 mmol), 2-chloro-5-fluoro-pyrimidine (15.0 g, 113 mmol, 14.0 mL), and K_2CO_3 (24.1 g, 174 mmol) in DMF (100 mL) was stirred at 60 °C for 48 h. The mixture was quenched with water and diluted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, 10–30% EtOAc in hexane) to give the title compound (3.1 g, 12.5 mmol, 14% yield) as an off-white solid; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.54 (s, 2H), 7.22 (t, *J* = 8.5 Hz, 1H), 6.58 (d, *J* = 8.6 Hz, 1H), 6.49 (s, 1H), 6.45–6.32 (m, 1H), 2.90 (s, 6H).

General Procedure of Palladium-Catalyzed Coupling Reaction. A mixture of 3-((2-chloropyrimidin-5-yl)oxy)-*N,N*-dimethylaniline (20.0 mg, 80.1 μ mol), amine (160 μ mol), BrettPhos Pd G3 (7.25 mg, 8.00 μ mol), BrettPhos (4.29 mg, 8.00 μ mol), KOTBu (27.0 mg, 240 μ mol), and DME (1 mL) was heated 100 °C for 2 h under microwave irradiation (Synthos 3000, 4x48MC). The reaction mixture was diluted with EtOAc (3 mL), quenched with H₂O (1 mL), and stirred for 2 min. The organic layer was separated, and the aqueous layer was then extracted with EtOAc (2 mL). The combined organic layer was evaporated at 60 °C. The residue was purified by preparative HPLC (Actus Triart C18, eluted with MeCN/10 mM NH₄HCO₃ aq 5:95 to 100:0). Pure fractions were combined and concentrated by blowing away with the air at 60 °C to afford product.

Preparation of $A\beta$ 42 Solutions. Biotinylated and non-biotinylated synthetic $A\beta$ 42 proteins were purchased from Anaspec (Fremont, CA). For AlphaLISA, $A\beta$ 42 was reconstituted in a minimal volume of 0.1% NH₄OH and then diluted with 20 mM HEPES buffer (pH 7.4) with 140 mM NaCl. Solubilized $A\beta$ 42 was spun at 20 000g for 10 min to remove aggregated material, and the protein concentration was estimated by BCA (Thermo Scientific). For $A\beta$ 42 aggregation studies, monomerized $A\beta$ 42 films (hexafluoro isopropanol, HFIP, treated) were prepared as in ref 34 and diluted in 20 mM Tris-HCl (pH 7.4) or 20 mM HEPES buffer (pH 7.4) with 140

mM NaCl. $A\beta$ 42 oligomers were prepared as described in ref 34. Briefly, monomerized $A\beta$ 42 films were reconstituted in DMSO by sonication for 10 min and incubated overnight with slow agitation (40 rpm) at 4 °C either in 20 mM Tris-HCl (pH 7.4) or 20 mM HEPES buffer (pH 7.4) with 140 mM NaCl. After overnight incubation, the solution was centrifuged at 20 000g for 10 min. The protein concentration was estimated by Pierce BCA protein assay kit (Thermo Scientific).

AlphaLISA. Various concentrations (0.1 – 100 μ M) of compounds were plated in white 384-well plates (Greiner) and were incubated with 10 nM biotinylated $A\beta$ 42 (Anaspec) and 1 nM fibrinogen for 30 min at room temperature (RT) in a final volume of 10 μ L assay buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20, and 0.1% BSA). The mixture was then incubated with anti-fibrinogen antibody (Dako), 20 μ g/mL streptavidin-conjugated donor, and protein A-conjugated acceptor beads (PerkinElmer) for 90 min at RT. Samples were read by a PerkinElmer EnVision plate reader. RU-505, which is a known inhibitor of $A\beta$ 42–fibrinogen interaction,²⁷ was used as a positive control. All the experiments were performed in triplicate. To determine IC₅₀ of compounds, the data were fitted to a sigmoidal dose–response equation ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / 1 + 10^{(\log IC_{50} - X) \cdot \text{Hill-coefficient}}$) using GraphPad Prism 4.

In Vitro Clotting (Turbidity) Assay. Turbidity assays were performed at RT in high binding 96-well plates (Fisher Scientific) in triplicate using a Spectramax Plus384 reader (Molecular Devices). For *in vitro* fibrin clot formation, fibrinogen (1.5 μ M) with or without $A\beta$ 42 oligomers (3 μ M) was mixed with thrombin (0.5 U/mL) and CaCl₂ (5 mM) in 20 mM HEPES buffer (pH 7.4) with 137 mM NaCl in a volume of 200 μ L. To test the efficacy of SEN1269 and its analogs in restoring the $A\beta$ 42-induced reduction in clot turbidity, clot formation was also initiated in the presence of these compounds (20 μ M) with $A\beta$ 42 oligomers (3 μ M). The effect of these compounds was also tested in normal fibrin clotting experiments without adding $A\beta$ 42 oligomers. Clot formation was monitored by measuring the change in turbidity at 450 nm over time.

Monitoring $A\beta$ 42 Aggregation Kinetics. Monomerized $A\beta$ 42 films (HFIP-treated) were reconstituted in DMSO by sonication as in ref 34. The solution was then diluted in 20 mM HEPES buffer (pH 7.4) with 140 mM NaCl and centrifuged at 14 000 rpm at 4 °C for 10 min to remove any aggregated species. The supernatant was collected, and $A\beta$ 42 concentration was estimated using the BCA method for protein quantification. The $A\beta$ 42 solution was kept on the ice and used for aggregation study reaction setup within 30 min. For amyloid aggregation kinetics, we used 96-well flat bottom microtiter black plates (Greiner), and the $A\beta$ 42 solution was added to the wells in the presence or absence of compounds. The final concentration of $A\beta$ 42 was 12.5 μ M, and the final concentration of compounds was 48 μ M in a total reaction mixture of 150 μ L. Because the stock solutions of these

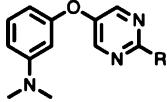
Table 1. Effect of Known A β Aggregation Inhibitors on the A β 42–Fibrinogen Interaction^a

Compound (Structure)	Name	Mol. Wt. (Da)	AlphaLISA (IC ₅₀)	95% CI (μ M)
	RS-0406	294.31	>100 μ M	-
	Tanshinone	294.34	>100 μ M	-
	SEN1576	408.43	>100 μ M	-
	Phenyl amino acetamide derivative	414.51	>100 μ M	-
	Aminostyryl benzofuran derivative	293.36	49.8 μ M	33.2 – 74.8
	4,5-Dianilino Pthalimide (DAPH)	329.35	43.2 μ M	36.4 – 51.4
	Nitrophenyl piperazinyl derivative	402.45	39.5 μ M	25.9 – 60.2
	Aminophenyl piperazinyl derivative	372.47	21.0 μ M	16.3 – 27.1
	SEN1269	322.36	17.8 μ M	16.0 – 19.9

^aStructures and other details of known A β aggregation inhibitors/ β -sheet breakers used in this study. These compounds were tested for their ability to inhibit the A β 42–fibrinogen interaction by AlphaLISA. The IC₅₀ of these compounds was determined and is presented with the structure of each compound. All AlphaLISA assays were performed in triplicate, and the 95% confidence interval (95% CI) was used as a statistical parameter.

compounds were in DMSO, the equivalent residual DMSO concentration was also added to A β 42 alone solution. To monitor the time dependent A β amyloid assembly, ThT dye³⁵ was added to each well to a final concentration of 33 μ M in the solution. Buffer alone in the presence and absence of compounds was also used with ThT as controls. The plate

was sealed with an adhesive film (Thin Seal) and incubated at 37 °C with a shaking speed of 25 rpm (Environ Orbital Shaker). Time dependent ThT fluorescence was recorded (once an hour) by quickly taking out the microtiter plate from the shaker and directly measuring the emission maxima at 480 nm with an excitation wavelength of 440 nm, using a

Table 2. Analogs Obtained by Right-Hand Side (RHS) Modifications of the SEN1269 Scaffold^a


Entry	R	ID	IC ₅₀ (μM)	95% CI (μM)	Entry	R	ID	IC ₅₀ (μM)	95% CI (μM)
1		SEN 1269	17.8	16.0 – 19.9	7		TDI-3099	29.1	24.8 – 34.2
2		TDI-2758	6.42	5.35 – 7.71	8		TDI-3071	69.0	60.7 – 78.7
3		TDI-3066	25.0	19.2 – 32.4	9		TDI-3078	43.5	39.1 – 48.3
4		TDI-3067	29.0	24.9 – 34.1	10		TDI-3277	9.9	7.74 – 12.7
5		TDI-3082	23.0	18.6 – 28.3	11		TDI-3275	6.2	3.16 – 12.0
6		TDI-2760	1.67	1.36 – 2.05					

^aThese analogs were tested for their efficacy in blocking the A β 42–fibrinogen interaction using AlphaLISA IC₅₀ was calculated. All AlphaLISA assays were performed in triplicate, and the 95% confidence interval (95% CI) was used as a statistical parameter.

Spectramax M2e plate reader (Molecular Devices). The microtiter plate was sealed after every measurement and incubated at 37 °C for a total of 26 h. All the experiments were performed in triplicate.

Scanning Electron Microscopy (SEM). Fibrin clots were formed using purified human fibrinogen (Calbiochem) on round glass coverslips at RT. Fibrin clots in the presence or absence of oligomeric A β 42 were formed as described above for the turbidity assay. To test the efficacy of SEN1269 and its analogs, these compounds (20 μM) or DMSO were preincubated with A β 42 oligomers (3.0 μM) for 10 min with slow shaking at RT. Fibrinogen (1.5 μM) was added into compound-A β 42 mixtures, and the reaction was initiated by adding thrombin (0.5 U/mL). Thirty minutes after adding thrombin, clots were gently washed twice with cold sodium cacodylate buffer (0.1 M, 2 min each) and fixed with 2% glutaraldehyde (on ice for 30 min). After serial dehydration by ice cold ethanol (20–100%), critical point dried fibrin clots were sputter-coated with gold palladium. Images were obtained using a LEO 1550 scanning electron microscope at the Electron Microscopy Resource Center at The Rockefeller University.

Transmission Electron Microscopy (TEM). For visualizing the morphology of A β 42 aggregates in the presence and absence of hit compounds, after 26 h of incubation, the undiluted A β 42 (12.5 μM) samples (in the presence and absence of compounds) were applied to glow-discharged CF200-Cu grids (Electron Microscopy Sciences), washed three times with ultrapure water, negatively stained with 2% uranyl acetate, and air-dried. Electron micrographs were

acquired using the FEI TECNAI G2 Spirit BioTwin transmission electron microscope (120 Kv) equipped with Gatan 4K × 4K digital camera in Electron Microscopy Resource Center at The Rockefeller University.

Kallikrein Activity Assay. To test the effect of TDI-2760 on A β 42 oligomer-induced kallikrein generation *in vitro*, A β 42 oligomers in the presence or absence of TDI-2760 were incubated in 20 mM HEPES buffer (pH 7.4) with 140 mM NaCl in a 96-well microtiter plate (Fisher Scientific). The plate was sealed with an adhesive sealing film (Thin Seal) and incubated at RT with a shaking speed of 25 rpm for 30 min. Buffer (in the presence or absence of TDI-2760) without A β 42 oligomers was also incubated. After 30 min, 10 nM purified human coagulation factor XII (FXII; Haematologic Technologies Inc.) and 10 nM human plasma prekallikrein (PPK; Enzyme Research Laboratories) were added to each well. Finally, chromogenic substrate S-2302 (Chromogenix) was added to each well to a final concentration of 0.67 mM in a 100 μL reaction mixture. Time dependent change in the absorbance was recorded by measuring the absorbance at 405 nm at RT with a Spectramax Plus 384 reader (Molecular Devices). In the reaction mixture of 100 μL, the final concentration of A β 42 oligomer was 1 μM and that of TDI-2760 was 40 μM. Experiments were performed in triplicate, and dextran sulfate (DXS) (0.1 μg/mL) was used as a positive control. DXS (0.1 μg/mL) in the presence of TDI-2760 (40 μM) was also used to check the specificity of TDI-2760. The rate of substrate (S-2302) conversion over time (30 min) was also calculated for

each sample by Softmax 6.1 (Molecular Devices) and represented as milliunits per min.

Statistical Analysis. All numerical values presented in graphs are mean \pm SEM. Statistical significance in Figure 5 was determined using Student's two-tailed *t* test. For all the AlphaLISA IC₅₀ analyses, 95% confidence interval (95% CI) was used as the statistical parameter.

RESULTS

Some A β Aggregation Inhibitors Can Block the A β –Fibrinogen Interaction. Biochemical analysis using AlphaLISA showed that the binding affinity of soluble A β oligomers to fibrinogen is nearly 10-fold higher than that of A β monomers.²⁷ Therefore, we hypothesized that A β aggregation inhibitors may indirectly minimize the A β –fibrinogen interaction by decreasing A β oligomerization. In addition, we hypothesized that some A β aggregation inhibitors may directly bind to A β ^{32,33} and disrupt the A β –fibrinogen interaction directly. Based on these hypotheses, we synthesized and evaluated a series of known A β aggregation inhibitors/ β -sheet breakers^{32,36–42} for their efficacy against the A β –fibrinogen interaction using AlphaLISA (Table 1). Some of the β -sheet breakers possessed efficacy against the fibrinogen–A β interaction (IC₅₀ values are shown in Table 1). However, the efficacy of these compounds (IC₅₀) varied considerably. For example, RS-0406, SEN1576, and tanshinoine^{36,41,42} showed almost no efficacy (IC₅₀ > 100 μ M), whereas SEN1269³² showed the best efficacy in blocking the A β 42–fibrinogen interaction (IC₅₀ = 17.8 μ M). Since SEN1269 has the highest inhibitory efficacy, we selected this compound for structure–activity relationship (SAR) study.

Chemical Modification of SEN1269 to Improve Its Inhibitory Efficacy against the A β 42–Fibrinogen Interaction. To improve the potency of SEN1269, we modified the chemical structure of the SEN1269 scaffold. SAR of the right-hand side (RHS) of SEN1269 is summarized in Table 2. The inhibitory activity of SEN1269 analogs against the A β 42–fibrinogen interaction was determined using AlphaLISA, and IC₅₀ values were calculated (Table 2). The methoxy analogs (Table 2; TDI-2758 entry 2, TDI-3066 entry 3, and TDI-3067 entry 4) were evaluated, and it was found that the substituent in the *meta* position (TDI-2758) was most effective among the three analogs (IC₅₀ = 6.42 μ M for entry 2 vs IC₅₀ = 25.0 and 29.0 μ M, for entries 3 and 4, respectively). Compared to the parent molecule, the substituents in the *ortho* and *para* positions actually led to decreased inhibitory activity against the A β 42–fibrinogen interaction. Furthermore, the phenyl analog (TDI-2760; Table 2 entry 6 and Figure 1A) showed much higher efficacy against this interaction (IC₅₀ = 1.67 μ M) in AlphaLISA (Table 2 and Figure 1B). In comparison to its parent molecule, the inhibitory efficacy (IC₅₀) of TDI-2760 was increased ~10-fold (17.8 μ M vs 1.67 μ M). RU-505 is a known inhibitor of the A β 42–fibrinogen interaction,²⁷ and thus we included it in our study as a positive control (Figure 1).

Next, a SAR study of the left-hand side (LHS) was also conducted (Table S1). The dimethylamino substituent on the phenyl group was crucial to disrupt the interaction (Table S1; entries 1 and 2), and the pyrrolidine analog, TDI-3222, led to enhanced activity (Table S1; entry 5; IC₅₀ = 3.12 μ M). This result would suggest that an electron-donating amino group leads to enhanced potency. To test this hypothesis, difluoropyrrolidine analog TDI-3906 was evaluated and revealed a decreased potency (Table S1; entry 6; IC₅₀ = 88.3 μ M). In addition, we tested several other LHS analogs

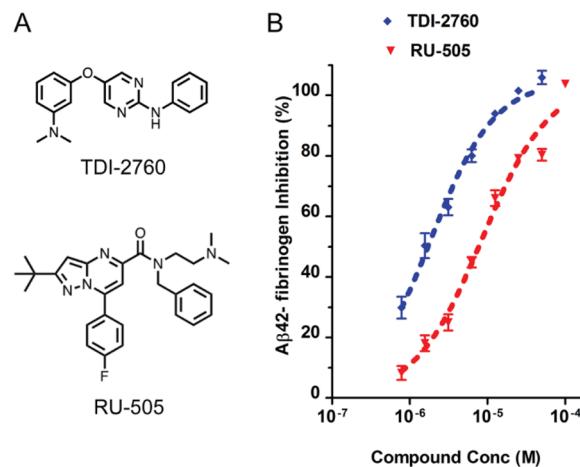


Figure 1. TDI-2760 blocks the interaction between A β 42 and fibrinogen in AlphaLISA. (A) The chemical structures of TDI-2760 and RU-505. (B) Representative AlphaLISA reading, showing the effect of TDI-2760 on the A β 42–fibrinogen interaction. IC₅₀ of TDI-2760 was determined, and RU-505 was used as a positive control since it inhibits the A β 42–fibrinogen interaction. The experiment was performed in triplicate, and mean \pm SEM values were plotted. Blue = TDI-2760; Red = RU-505.

including morpholino and mono-N-methyl analog, but only the N-methyl analog led to improved potency effectively compared with the original compound (Table S1; entry 10, IC₅₀ = 3.76). Based on these preliminary SAR results, we found that TDI-3222 and TDI-3256, which were obtained by RHS modifications, have better inhibitory efficacy against the A β 42–fibrinogen interaction (IC₅₀ = 3.12 μ M and 3.76 μ M, respectively). Overall, the TDI-2760, which was obtained by RHS modifications, exhibited the most potent efficacy against the A β 42–fibrinogen interaction as its IC₅₀ value was 10-fold lower than its parent molecule, SEN1269 (1.67 μ M vs 17.8 μ M, respectively).

Validating the Inhibitory Efficacy of SEN1269 and Its Analogs against the A β 42–Fibrinogen Interaction Using an *in Vitro* Clotting Assay. As the interaction between fibrinogen and A β induces a structurally abnormal fibrin clot,^{15,18,27} we tested whether SEN1269 and its analogs (TDI-3222, TDI-3256, and TDI-2760) can restore A β -induced altered thrombosis *in vitro*. We also included TDI-2758 (entry 2, Table 2) in the assays, since this analog also showed improved inhibitory efficacy against the A β –fibrinogen interaction by AlphaLISA.

During clot formation, fibrinogen molecules are cleaved by thrombin and associate into a fibrin network, which scatters light and causes the solution to increase in turbidity. Thus, the kinetics of turbidity can be used as a read-out to examine fibrin clot formation.²⁷ Each compound (20 μ M) or vehicle (0.4% DMSO) was incubated with purified human fibrinogen in the presence or absence of oligomeric A β 42. Fibrin clot formation was analyzed by measuring the turbidity immediately after adding thrombin to the mixture. The maximum turbidity of the fibrin clot decreased in the presence of A β 42, since it alters fibrin clot structure (Figure 2A–D). In the *in vitro* clotting assay, SEN1269 further decreased the turbidity of the clot formed in the presence or absence of A β 42 (Figure 2A). This result indicates that SEN1269 also altered normal fibrin clot structure, which was undesirable. In contrast, TDI-2758 and TDI-3256 did not alter the turbidity of the normal fibrin clot

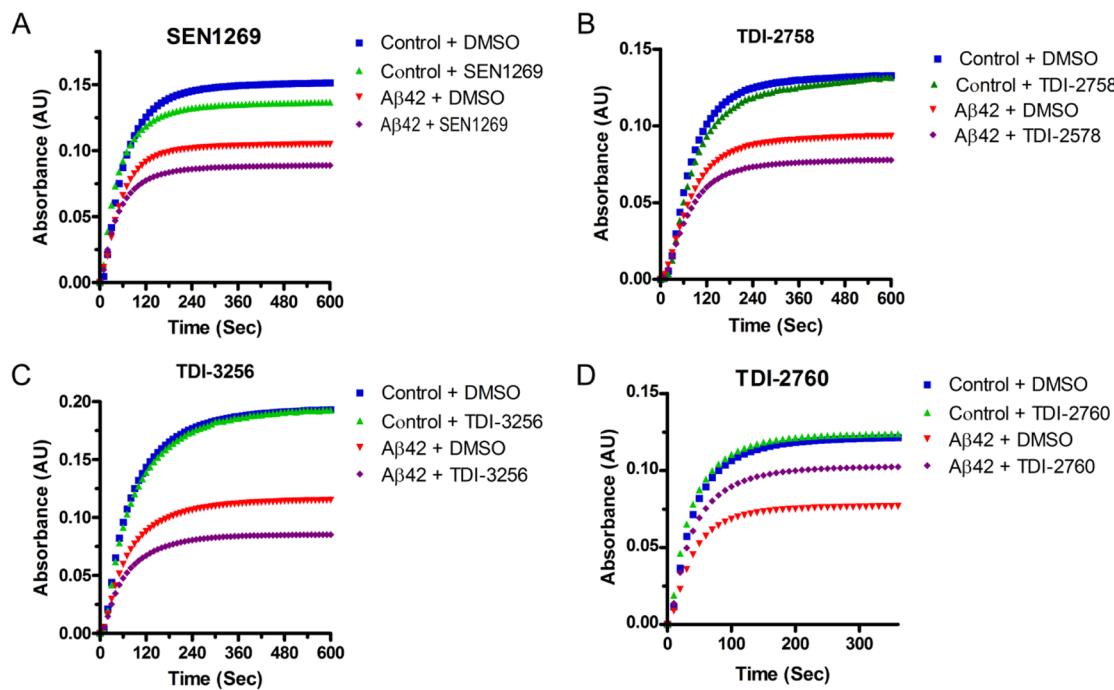


Figure 2. TDI-2760 restores the $\text{A}\beta 42$ -induced decrease in fibrin clot turbidity without affecting normal clotting in an *in vitro* clotting assay. Individual test compounds or DMSO (vehicle) was incubated with fibrinogen in the presence or absence of $\text{A}\beta 42$ ($3 \mu\text{M}$), followed by addition of thrombin and CaCl_2 . Fibrin clot formation was assessed by measuring time-dependent turbidity changes at 450 nm ($n = 3$ experiments/compound). (A) SEN1269 decreased the clot turbidity of the normal clot formed in the absence of $\text{A}\beta 42$ (green vs blue) as well as the turbidity of the clot formed in the presence of $\text{A}\beta 42$ (purple vs red). (B) In the presence of TDI-2758, the turbidity of clots formed with $\text{A}\beta 42$ was decreased (purple vs red). (C) In the presence of TDI-3256, the turbidity of clots formed with $\text{A}\beta 42$ was also decreased (purple vs red). (D) TDI-2760 did not alter the turbidity of the normal clot (blue vs green) but restored the $\text{A}\beta 42$ -induced decrease in turbidity during fibrin clot formation (purple vs red).

formed in the absence of $\text{A}\beta 42$ (Figure 2B,C, green), but clot turbidity was further decreased in the presence of both $\text{A}\beta 42$ and TDI-2758/TDI-3256 (Figure 2B,C, purple). This result was the opposite of what we expected, as these analogs showed an inhibitory effect on the $\text{A}\beta 42$ –fibrinogen interaction in AlphaLISA. TDI-3222 did not show any effect in the turbidity assay due to its color causing a high background (data not shown).

In contrast to the other compounds, phenyl analog TDI-2760 was very effective in the turbidity assay as it partially restored the $\text{A}\beta 42$ -induced abnormal fibrin clot formation (Figure 2D, purple vs red). Additionally, TDI-2760 did not alter the turbidity of the normal clot in the absence of $\text{A}\beta 42$ (Figure 2D, green vs blue). This observation indicates that TDI-2760 specifically restores the $\text{A}\beta$ -induced decrease in turbidity during fibrin clot formation without affecting normal thrombosis. In conclusion, TDI-2760 effectively inhibits the $\text{A}\beta$ –fibrinogen interaction and partially restores $\text{A}\beta 42$ -induced abnormal fibrin clot formation.

The results of the turbidity assay were further validated by analyzing *in vitro* fibrin clots using scanning electron microscopy (SEM). Fibrin clots were prepared from purified fibrinogen in the presence of CaCl_2 and thrombin, and SEM images of fibrin clots showed a typical three-dimensional mesh of elongated fibrin threads (Figure 3A). However, in the presence of $\text{A}\beta 42$ oligomers, the fibrin threads were relatively thinner (Figure 3B) and several clumped structures were observed, indicating that the architecture of the fibrin clot was altered in the presence of $\text{A}\beta 42$ (Figure 3B). Consistent with the turbidity assay, TDI-2760 partially restored the $\text{A}\beta 42$ -induced abnormal fibrin clot structure as evidenced by fewer

clumps and relatively thicker fibrin threads under SEM (Figure 3C). However, the SEM analysis of fibrin clots in the presence of $\text{A}\beta 42$ and SEN1269 (Figure 3D) or TDI-2758 (Figure 3E) suggested that SEN1269 and TDI-2758 failed to restore the $\text{A}\beta 42$ -induced clot abnormality. In fact, clots formed in the presence of $\text{A}\beta 42$ with SEN1269 or TDI-2758 showed more clumps and compromised clot architecture more severely than clots formed in the presence of $\text{A}\beta 42$ oligomers only (Figure 3D,E). This finding was consistent with the results obtained from the turbidity assay, in which SEN1269 and TDI-2758 further decreased the turbidity of the fibrin clots formed in the presence of $\text{A}\beta 42$ (Figure 2A,B).

Even though TDI-2760 did not affect normal thrombosis in the turbidity assay (Figure 2D), we further tested whether TDI-2760 affects thrombin proteolytic activity or blood coagulation, using a chromogenic substrate assay and activated partial thromboplastin time (aPTT) test, respectively. Our assay results showed that TDI-2760 did not alter the activity of thrombin in the chromogenic substrate assay (Figure S1) and did not have any effect on the coagulation profile of human pooled normal plasma in a microtiter plate based-aPTT test (Figure S2).

TDI-2760 Effectively Inhibits $\text{A}\beta 42$ Aggregation. SEN1269 was not effective in reversing the $\text{A}\beta 42$ -induced decrease in clot turbidity and altered clot structure (Figure 2A and Figure 3D). However, SEN1269 has been shown to be a potent $\text{A}\beta$ aggregation inhibitor,³² and it also effectively inhibited $\text{A}\beta 42$ aggregation in our study (Figure S3). Therefore, we investigated if SEN1269 phenyl analog, TDI-2760, also retained this inhibitory efficacy toward $\text{A}\beta 42$ aggregation *in vitro*. The time-dependent $\text{A}\beta 42$ aggregation kinetics were

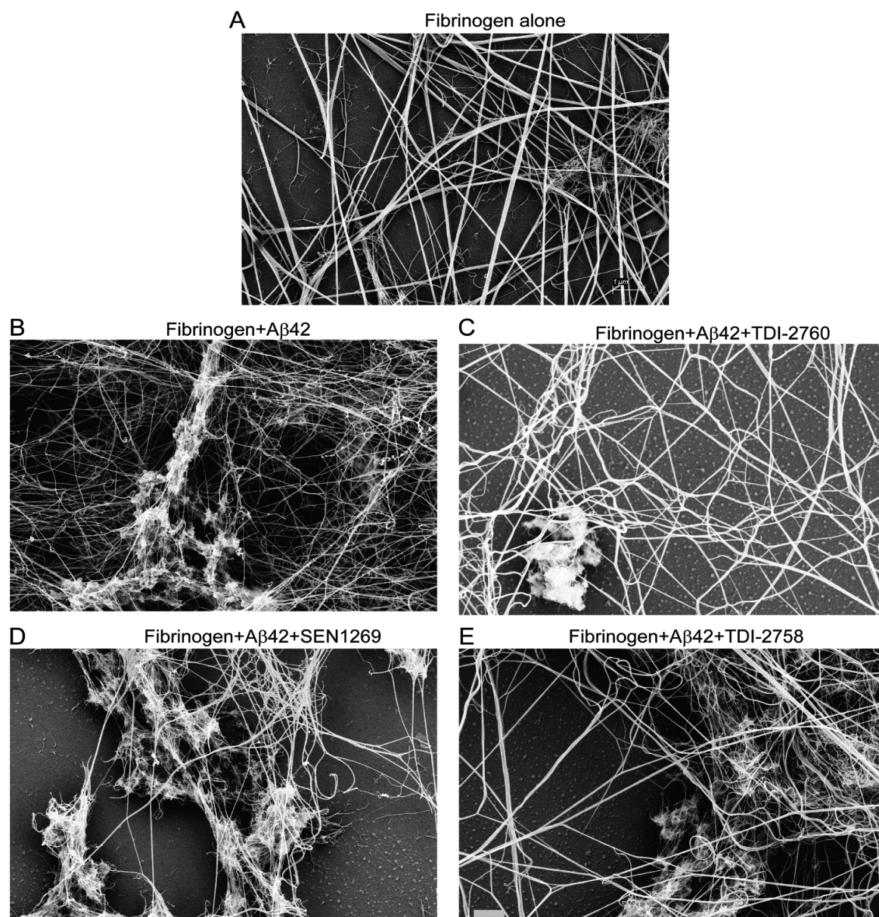


Figure 3. SEM analysis of the effect of hit compounds on $\text{A}\beta 42$ -induced abnormal fibrin clot structure. Individual compounds ($20 \mu\text{M}$) or DMSO (vehicle) was incubated with fibrinogen ($1.5 \mu\text{M}$) in the presence or absence of $\text{A}\beta 42$ oligomers ($3 \mu\text{M}$), and clot formation was initiated by adding thrombin into the mixture. Fibrin clot structures were analyzed by SEM. SEM images of clots formed by (A) Fibrinogen alone, (B) Fibrinogen + $\text{A}\beta 42$, (C) Fibrinogen + $\text{A}\beta 42$ + TDI-2760, (D) Fibrinogen + $\text{A}\beta 42$ + SEN1269, (E) Fibrinogen + $\text{A}\beta 42$ + TDI-2758. SEM analysis revealed that $\text{A}\beta 42$ oligomers altered the fibrin clot structure, and TDI-2760 partially restored the $\text{A}\beta 42$ -induced structural abnormality of fibrin clot while SEN1269 and TDI-2758 did not. Scale bar is $1 \mu\text{m}$.

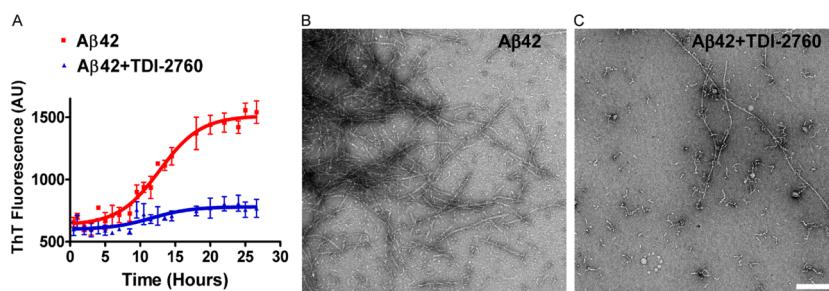


Figure 4. TDI-2760 inhibits $\text{A}\beta 42$ fibrilization. $\text{A}\beta 42$ aggregation in the presence and absence of TDI-2760 was examined by monitoring amyloid aggregation kinetics by ThT fluorescence. (A) ThT fluorescence was monitored over time, measuring the emission maxima at 480 nm with an excitation wavelength of 440 nm . ThT fluorescence of the $\text{A}\beta 42$ alone (red) showed a time-dependent increase and displayed a sigmoidal growth curve, which is a typical characteristic of amyloid aggregation. In the presence of TDI-2760 (blue), the $\text{A}\beta 42$ -induced time-dependent increase of ThT fluorescence was significantly reduced, suggesting that TDI-2760 strongly inhibits $\text{A}\beta 42$ fibrilization. The experiment was performed in triplicate, and the graph is presented as mean \pm SEM value. (B, C) After 26 h, these samples were visualized by TEM. TEM images of $\text{A}\beta 42$ alone (B) showed predominantly fibrillar species along with some oligomers. The fibrillar and oligomeric species of $\text{A}\beta 42$, however, were substantially decreased in the presence of TDI-2760 (C), suggesting the inhibitory efficacy of this compound against $\text{A}\beta 42$ aggregation. Scale bar is 100 nm .

probed using an amyloid-specific dye, thioflavin T (ThT).³⁵ The typical amyloid aggregation process (when probed using ThT) shows a sigmoidal growth curve with lag phase, log phase, and stationary phase. The stationary phase (with a plateau) usually represents the completion of the amyloid fibrilization process.⁴³ The ThT fluorescence of $\text{A}\beta 42$ ($12.5 \mu\text{M}$) in the presence and absence of TDI-2760 ($48 \mu\text{M}$) was recorded at different time intervals. The ThT fluorescence data of $\text{A}\beta 42$ alone showed a typical sigmoidal curve, characteristic of the amyloid aggregation pathway (red in Figure 4A).

In contrast, $\text{A}\beta 42$ in the presence of TDI-2760 did not show such characteristics, and the ThT fluorescence was significantly

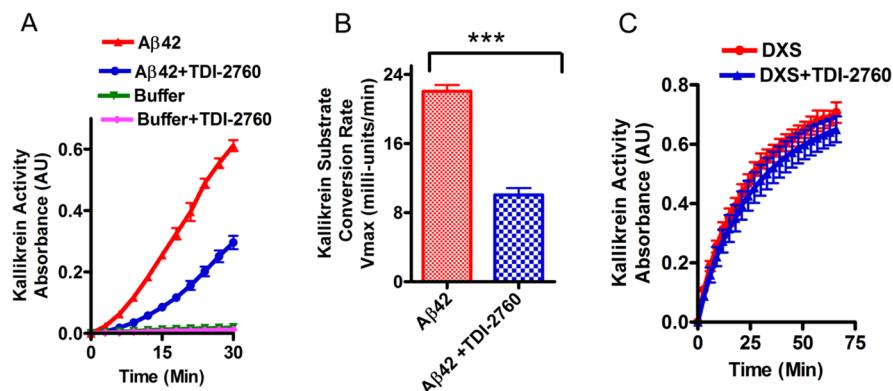


Figure 5. Effect of TDI-2760 on $\text{A}\beta_{42}$ -mediated contact activation. (A) Purified human coagulation factor XII (FXII) and human plasma prekallikrein (PPK) were used in the assay, and kallikrein activity was triggered by adding $\text{A}\beta_{42}$ oligomers incubated in the presence or absence of TDI-2760. The final concentration of $\text{A}\beta_{42}$ oligomers was $1 \mu\text{M}$ and TDI-2760 was $40 \mu\text{M}$ in the reaction. Buffer alone (green) and buffer with TDI-2760 (purple) showed essentially no kallikrein activity. $\text{A}\beta_{42}$ alone (red) significantly increased kallikrein activity, while TDI-2760 with $\text{A}\beta_{42}$ (blue) led to a significant decrease in the amount of kallikrein release. (B) V_{max} was calculated from the assay in panel A. Preincubating $\text{A}\beta_{42}$ oligomers with TDI-2760 prior to this assay led to a significantly lower substrate (S-2302) conversion rate (V_{max}) ($p < 0.001$). The experiment was performed in triplicate, and mean \pm SEM values are plotted. (C) Dextran sulfate (DXS, red, $0.1 \mu\text{g}/\text{mL}$), a positive control for kallikrein activation, was not influenced by preincubation with TDI-2760 (blue, $40 \mu\text{M}$), indicating TDI-2760's specificity for $\text{A}\beta_{42}$. Statistical significance was determined by nonparametric unpaired (two-tailed) t -test. $p \leq 0.001$ was designated as ***.

lower (blue in Figure 4A). This result indicates minimal $\text{A}\beta_{42}$ amyloid fibril formation in the presence of TDI-2760. ThT fluorescence of buffer in the presence and absence of TDI-2760 without $\text{A}\beta_{42}$ addition remained low during the incubation period (data not shown). To confirm this result, we used transmission electron microscopy (TEM) to examine the end products of $\text{A}\beta_{42}$ aggregation in the presence or absence of TDI-2760 after 26 h incubation. TEM of $\text{A}\beta_{42}$ showed mainly fibrillar structures with few oligomers (Figure 4B), consistent with high ThT fluorescence (Figure 4A). However, TEM of $\text{A}\beta_{42}$ in the presence of TDI-2760 showed very few fibrillar structures (Figure 4C), consistent with low ThT fluorescence (Figure 4A). The ThT fluorescence data and TEM analysis clearly revealed that TDI-2760 effectively inhibits $\text{A}\beta_{42}$ aggregation. This observation suggests that removing the hydroxyl ($-\text{OH}$) group improves the inhibitory efficacy of SEN1269 against the $\text{A}\beta_{42}$ –fibrinogen interaction without diminishing its ability to inhibit $\text{A}\beta_{42}$ aggregation.

Effect of TDI-2760 on $\text{A}\beta_{42}$ -Mediated Initiation of Contact Activation System. Induction of the coagulation factor XII (FXII)-driven contact activation pathway can be initiated by $\text{A}\beta_{42}$,^{17,44–47} leading to cleavage of coagulation factor XI (FXI) and blood clotting and activation of plasma prekallikrein (PPK) and release of bradykinin from high molecular weight kininogen (HK).⁴⁸ This activation could play a role in the vascular and inflammatory aspects of AD pathology by launching both prothrombotic and proinflammatory pathways.²⁵ Since SEN1269 binds to $\text{A}\beta_{42}$ in a surface plasmon resonance experiment,³² TDI-2760, the phenyl analog of SEN1269, may bind to $\text{A}\beta_{42}$, since it showed strong inhibition against the $\text{A}\beta_{42}$ aggregation process (Figure 4). We speculated that if TDI-2760 interacts with $\text{A}\beta_{42}$, it may also affect $\text{A}\beta$'s activation of FXII and subsequent cleavage of FXI to FXIa or PPK to kallikrein or both. To test this hypothesis, we measured the *in vitro* kallikrein activity using purified human coagulation components (FXII and PPK) and a chromogenic substrate, S-2302. $\text{A}\beta_{42}$ oligomers in the presence and absence of TDI-2760 were tested for their potential to activate FXII and thus produce kallikrein. Kallikrein then can cleave its chromogenic substrate S-2302, which can be quantified by measuring

absorbance at 405 nm .¹⁷ $\text{A}\beta_{42}$ oligomers ($1 \mu\text{M}$) showed high kallikrein activity in the reaction mixture of purified coagulation components and the S-2302 substrate, as indicated by high absorbance at 405 nm (red in Figure 5A).

However, $\text{A}\beta_{42}$ oligomers incubated in the presence of TDI-2760 ($40 \mu\text{M}$) showed significantly less absorbance at 405 nm compared to $\text{A}\beta_{42}$ oligomers alone (blue in Figure 5A). The buffer in the presence and absence of TDI-2760 ($40 \mu\text{M}$) without $\text{A}\beta_{42}$ addition showed insignificant kallikrein activity (purple and green in Figure 5A). This suggests that binding of TDI-2760 to $\text{A}\beta_{42}$ oligomers lessens the ability of $\text{A}\beta$ to induce FXII activation and its subsequent generation of kallikrein. Consistent with this result, the rate of S-2302 substrate conversion was also significantly lower in the $\text{A}\beta_{42}$ oligomers incubated with TDI-2760 sample (22.06 ± 0.73 vs 10.06 ± 0.78 milliunits per min, respectively) (Figure 5B). Dextran sulfate (DXS, MW 500 KDa)¹⁷ was used as a positive control and showed high kallikrein activity (red in Figure 5C). Additionally, TDI-2760 ($40 \mu\text{M}$) did not alter the kallikrein activity induced by DXS ($0.1 \mu\text{g}/\text{mL}$) (blue in Figure 5C), indicating the specificity of TDI-2760 toward $\text{A}\beta_{42}$. These data suggest that TDI-2760 is effective at blocking the $\text{A}\beta_{42}$ -induced activation of the FXII-mediated contact system.

DISCUSSION

Several studies indicate that $\text{A}\beta$ aggregation initiates a cascade of molecular events, eventually leading to neuronal death and dementia in AD.^{1,2,6–8} Therefore, targeting $\text{A}\beta$ aggregation has increasingly become a focus of a potential therapeutic approach for AD.^{32,33,38,49–54} Despite tremendous efforts, drug development of $\text{A}\beta$ aggregation inhibitors has proven very difficult, and the results of ongoing clinical trials of those inhibitors are mostly inconclusive.^{55,56} In this study, we have shown that some $\text{A}\beta$ aggregation inhibitors^{32,36–40} have the intrinsic ability to disrupt the $\text{A}\beta$ –fibrinogen interaction (Table 1). These characteristics together would be beneficial not only in altering $\text{A}\beta$ aggregation-related toxicity but also in modulating vascular abnormalities that arise from the $\text{A}\beta$ –fibrinogen interaction in AD.

Because our previous work indicated that $\text{A}\beta$ oligomers have stronger affinity for fibrinogen than monomers, we tested whether $\text{A}\beta$ aggregation inhibitors could inhibit the $\text{A}\beta$ –fibrinogen interaction in the present study.²⁷ However, our results in Table 1 suggest that $\text{A}\beta$ aggregation inhibition itself is not sufficient to inhibit the $\text{A}\beta$ –fibrinogen interaction. For example, RS-0406, SEN1576, and tanshinone,^{36,41,42} all of which can strongly inhibit $\text{A}\beta$ aggregation and its toxicity, showed negligible efficacy against the $\text{A}\beta$ –fibrinogen interaction ($\text{IC}_{50} > 100 \mu\text{M}$) (Table 1). In addition, since we used the oligomeric form of $\text{A}\beta$ in turbidity assays (Figure 2) and kallikrein activity assays (Figure 5), the ability to inhibit $\text{A}\beta$ aggregation, such as these compounds exhibit, may be inconsequential in affecting the result of both assays. Moreover, the duration of both assays (including the incubation time for compound– $\text{A}\beta$ mixtures) was less than an hour. It is very unlikely that $\text{A}\beta$ aggregation status affects the result of both the assays within an hour, because $\text{A}\beta$ fibrillation requires several hours at 37 °C (Figure 4A). Therefore, we expect that $\text{A}\beta$ binding affinity of compounds could be an important factor in inhibitory efficacy against the $\text{A}\beta$ –fibrinogen interaction. In line with this, SEN1269 (5-aryloxyprymidine) was shown to bind $\text{A}\beta42$ and subsequently block $\text{A}\beta42$ ’s β -pleated sheet assembly and toxicity.³² Although it is not clear whether SEN1269 or its analog compounds bind directly to fibrinogen, we found that TDI-2760 does not affect normal thrombosis in the turbidity assay (Figure 2D) or blood coagulation in an activated partial thromboplastin time (aPTT) test using human plasma (Figure S2). These results suggest that TDI-2760 does not have any effect on the activity of coagulation proteases. In addition to fibrin polymer formation via coagulation cascade, fibrin(ogen) is also involved in other activities, such as binding to platelets via $\alpha\text{IIb}\beta\text{3}$ or leukocytes via Mac-1. This type of fibrin(ogen) activity is important for hemostasis or innate immunity.^{57,58} Therefore, to become an effective therapeutic molecule, the influence of TDI-2760 on fibrin(ogen) binding to platelets or leukocytes needs to be examined in future studies.

The contact activation system, driven by FXII, can also launch a proinflammatory pathway that leads to the release of bradykinin upon cleavage of HK.^{17,48} A significantly higher level of HK cleavage is reported in AD patient plasma.¹⁷ Furthermore, it was recently shown that modulating the innate immunity response by knocking out the complement component C3 led to neuroprotection and improved cognitive function in AD mice.⁵⁹ These complement C3-knockout AD mice performed better on learning and memory tests despite having more cerebral $\text{A}\beta$ deposition. The reduced level of proinflammatory markers in these AD mice highlights the important role of inflammation in AD and suggests that targeting the inflammatory pathway could be highly beneficial in treating this disease.⁵⁹ Consistent with this finding, our group has shown that reducing neuroinflammation by depleting FXII improves the cognitive function of AD mice.²⁵

$\text{A}\beta42$ can activate FXII,^{17,44–46} which leads to the cleavage of PPK into kallikrein.⁴⁸ AD patient plasma has increased levels of kallikrein-like activity compared to that of nondemented control individuals, further suggesting the crucial role of inflammation in AD pathology.^{17,19} TDI-2760 lessens the $\text{A}\beta42$ -driven activation of FXII and subsequent release of kallikrein *in vitro* (Figure 5). All these results suggest that TDI-2760 not only alters $\text{A}\beta42$ aggregation but also modulates the other molecular activities of $\text{A}\beta42$, such as fibrinogen interaction and contact system activation. These molecular

events are critical in driving vascular and inflammatory pathologies in AD. Therefore, the present study suggests that molecules that can participate in minimizing $\text{A}\beta42$ -driven activation of the contact system and can inhibit the $\text{A}\beta42$ –fibrinogen interaction would be promising drug candidates for the treatment of AD patients with vascular and inflammatory defects.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.biochem.7b01214](https://doi.org/10.1021/acs.biochem.7b01214).

Methods for thrombin activity assay and aPTT test, SAR analysis of SEN1269 analogs by LHS modifications, effect of TDI-2760 on thrombin activity and aPTT, and effect of SEN1269 on $\text{A}\beta42$ aggregation (PDF)

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Author Contributions

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Author Contributions

P.K.S. performed experiments, analyzed the results, and wrote the manuscript. M.K. designed and conducted chemical synthesis, analyzed the data, and wrote the manuscript. H.B.R. performed experiments. G.N. performed experiments. T.Y. performed experiments. M.A.F. helped design the experiments and helped analyze the results. E.H.N. helped analyze the data and write the manuscript. S.S. helped analyze the results and write the manuscript. K.A. designed and performed chemical synthesis and wrote the manuscript. H.J.A. designed and performed experiments, analyzed the results, and wrote the manuscript. All the authors edited and approved the final version of the manuscript.

Funding

This work was supported by the Alzheimer’s Drug Discovery Foundation and Robertson Therapeutic Development Fund for H.A., NIH grant NS50537, the Tri-Institutional Therapeutics Discovery Institute, Alzheimer’s Drug Discovery Foundation, Rudin Family Foundation, and John A. Herrmann for S.S.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank The Rockefeller University’s High-Throughput and Spectroscopy, and Electron Microscopy Resource Centers for access to experimental equipment and technical support. We thank Zu-Lin Chen and members of the Strickland lab for helpful discussion.

ABBREVIATIONS

$\text{A}\beta$, β -amyloid; AD, Alzheimer's disease; aPTT, activated partial thromboplastin time; BSA, bovine serum albumin; CAA, cerebral amyloid angiopathy; CI, confidence interval; DMSO, dimethyl sulfoxide; DXS, dextran sulfate; FXI, coagulation factor XI; FXII, coagulation factor XII; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFIP, hexafluoro isopropanol; HK, high molecular weight kininogen; HPLC, high-performance liquid chromatography; IC₅₀, half maximal inhibitory concentration; LHS, left-hand side; PPK, plasma prekallikrein; RHS, right-hand side; SAR, structure–activity relationship; SEM, scanning electron microscopy; TEM, transmission electron microscopy; ThT, thioflavin T

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