Small Molecule Interactions with Self-Assembling Peptides

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ABSTRACT

Self-assembling peptides, such as amyloid beta and heat shock protein 27 (Hsp27), contribute to pathologies and essential cell functions. Progressive aggregation of amyloid beta (Aβ) peptide is responsible for the pathologies of Alzheimer's disease (AD). Recent evidence suggests that Aβ aggregates are found in a variety of conformations, including globular oligomers and linear fibrils, which oligomers are found to be more neurotoxic than other Aβ conformations. Meanwhile, Hsp27 functions as a chaperone in oligomeric structure but works as an apoptotic regulator in its monomeric state. Hsp27 is neuroprotectant and has been shown to diminish neuronal aggregates. Hence, substances that interact selectively with different Aβ conformations and that promote Hsp27 oligomeric formation would be useful tools in developing chemical probes to study the molecular recognition structures that are essential in Aβ peptide and Hsp27 self-assembly and in developing potential therapeutics for AD. To identify substances that selectively impact different Aβ conformations, we screened 24 known Aβ ligands and WKLVFF peptide against Aβ oligomers and fibrils at different time points using fluorescence assays. We discovered that three tetracyclines specifically inhibit fibril aggregation at all time points, but complete inhibition of oligomer formation only occurs when compounds were added prior to aggregation. The fluorescence of WKLVFF peptide was also selectively quenched (55.44±4.08%) in the presence of fibrils, but slight increased (8.40±3.13%) in oligomers. These results suggest that tetracyclines and WKLVFF may bind to a hydrophobic domain that is critical in Aβ aggregation and this domain is exposed distinctively in fibril and in mature oligomers. In a parallel study, we aimed to identify small molecule modulators of Hsp27 activity by screening 35 compounds using turbidity assay. We observed that several hydrophobic or aromatic compounds effectively induce Hsp27 oligomerization. This result proposes that these sterols

interact with the conserved hydrophobic motif on Hsp27 and modulate its surface hydrophobicity. The understanding of structures that are essential in Aβ peptide and Hsp27 self-assembly permits further rational design of small molecules or biologics that could selectively probe for specific conformations of Aβ and manipulate the aggregation of Aβ peptide and Hsp27.

INTRODUCTION

Molecular self-assembly is exhibited in self-assembling peptides to form quaternary structures and the mechanism is evident in amyloid beta (Aβ) and Heat shock protein 27 (Hsp27). Intermolecular recognition and self-assembly is often achieved through the association between hydrophobic residues on the surface of peptides [1]. Therefore, the protein sequence that enables self-assembly could be investigated in both *in vivo* and *in vitro*. Small molecules that binds to these proteins and impacts the self-assembly properties could be used to manipulate the formation of supramolecular structure of the peptides and to control the cell function. We investigated the role of small molecules in impacting protein aggregations in amyloid beta (Aβ) and Heat shock protein 27 (Hsp27) models.

Interaction between Small Molecules or Short Peptide Fragment (WKLVFF) and Different Conformations of Amyloid beta Peptides

Alzheimer's disease (AD) is a neurodegenerative disorder that affects over 30 million individuals worldwide. AD is characterized by age-dependent aggregation of Aβ peptide in the brain [2,3]. The Aβ peptide is a 40 or 42 amino acid proteolytic fragment of amyloid precursor protein (APP) [4]. Upon cleavage of APP, Aβ forms β-sheets that promote subsequent aggregation. Both *in vitro* and *in vivo*, Aβ monomers will self-assemble into different conformations of higher order structures, such as low molecular weight (LMW) species (such as dimers and trimers), globular oligomers, and linear fibrils [2,5]. Recent studies in cell culture and animal models show that the LMW species and oligomers are more neurotoxic than the elongated fibrils that are observed in

late-stage Alzheimer's disease patients [6-11]. For instance, Aβ oligomers that could permeate lipid membrane [12-14] are shown to inhibit long-term potentiation and disrupt memory in AD mouse model [15,16]. Moreover, several hydrogen-deutarium exchange (HDE) and NMR studies suggested that oligomers consist of predominantly parallel beta-sheets that are packed into a spherical structure with diameter of 30-50nm [11, 17]. Oligomers are also found to be more solvent and less stable compared to fibrils [18]. All these findings indicate that fibrils and oligomers have different exposed structures that render each Aβ conformations its unique structural and biological properties. However, molecular structures of these distinct amyloid conformations and the conformational conversions have not been established. Moreover, there are not many reagents available to distinguish these structures [19]. Hence, it is important to identify Aβ peptide ligands that can recognize the difference of molecular structures between oligomers and fibrils. In this effort, we pursued two different approaches: identifying small molecules that selectively bind to different Aβ morphologies and investigating the binding selectivity of peptide-based ligand.

Small molecule screen

Currently histological dye with spectral properties such as Congo Red (CR), curcumin and thioflavin T (ThT) are usually used to monitor and quantify the growth of amyloid fibrils. However, these reagents lack the ability to distinguish the intermediate oligomers and fully-developed fibrils [20,21,55] because they interact with the similar pleated β-sheets in both Aβ conformations [22]. Necula *et. al.* showed that a number of small molecules that could selectively inhibit Aβ oligomerization or fibrilization [19]. Therefore, we hypothesize that small molecules that bind with the distinct exposed structures on oligomers and fibrils could be utilized

as probes to distinguish the Aβ species and to investigate their difference in molecular structures. We screened 24 structurally diverse small molecules, which are all known Aβ ligands, against isolated Aβ oligomers and fibrils at different time points using fluorescence assay. We discovered that three tetracycline derivatives inhibit fibril aggregation at all time points, but complete inhibition of oligomerization only occurs when compounds were added prior to aggregation. These results suggested that tetracyclines bind to a domain that is essential in Aβ aggregation that is likely to be hidden from solvent upon mature oligomer formation. This result supported previous study that showed tetracycline inhibited amyloid formation in prion peptide [23]. Through NMR spectroscopy, aromatic groups of tetracycline was found to interact with hydrophobic side-chain of Val121-122 and Leu125 on PrP106-126, which is a domain that is crucial in developing β-sheets that self-assemble into prion amyloid fibrils. In Aβ peptide, residues 16 to 20 (KLVFF motif) have been shown to be essential for β-sheet formation [24] and this may suggest that tetracycline binds to hydrophobic groups in this motif and that the binding site is masked when Aβ peptide is packed into globular oligomers.

Short peptide fragment (WKLVFF) screen

KLVFF short pentapeptide fragment can bind to similar residues on full-length Aβ peptide [25], hence blocking the β-strands and inhibiting fibril formation. Recent studies that adopted alanine substitution approach showed that amino acids Lys16, Leu17, and Phe20 are critical in binding with adjacent Aβ peptides and in inhibiting of Aβ fibrillization. The highly conserved phenylalanine residues (FF motif) [26] in this short functional fragment may suggest that these high affinity and selectivity residues are essential in directing molecular recognition, most probably through π-stacking interactions among the aromatic group [27-28], and self-assembles

into full-length Aβ peptide. Previous study suggested that hydrophobic core of LMW Aβ aggregates and oligomer has distinct location and configuration compared to fibrils [17]. To adopt more direct approach to study the role and exposure of highly conserved hydrophobic motif in amyloid formation, we also tested the binding specificity of short Aβ peptide fragment, KLVFF in LMW aggregates, oligomers and fibrils. The pentapeptide was synthesized through solid-phase protein synthesis and Trp was added to the N-terminal of the sequence so that the pentapeptide could be screened with Aβ oligomers and fibrils using fluorescence assay. We found that the fluorescence of WKLVFF peptide underwent 12nm blue shift and was quenched upon interacting specifically with fibrils or LMW species, but not oligomers. The blue shift suggested that positive charge of Lys16 was exposed in fibril conformation [29] and that the KLVFF hydrophobic core was exposed in fibrils or early stages of oligomers, but diminishes upon maturation of oligomers. This result, together with the findings of small molecule screening, showed that the highly conserved hydrophobic residues may be exposed differently in each Aβ conformations.

Interaction between Small Molecules and Monomeric Hsp27.

Heat shock proteins (HSP) family of proteins consists of a group of heat-responsive cell stress proteins that have chaperone functions, usually following thermal stress. HSPs are divided into subfamilies based on molecular weight, domain conservation and function, including small HSPs (sHSPs). sHsps, such as Hsp27, are ubiquitously expressed and characterized by the presence of α-crystallin domain [30], a highly conserved C-terminal domain that contains β-pleated sheets, that allows formation and stabilization of multimeric Hsp27 [31]. Hsp27 expression can be

rapidly induced by variety of factors, including stress, mitosis or environmental changes, via several transactivation domains. Hsp27 has many cellular and molecular functions depending on its state. Newly transcribed, non-phosphorylated Hsp27 forms large oligomeric structures that are prerequisite to chaperone activity [32]. Upon different stimuli, N-terminal of Hsp27 can be phosphorylated, leading to disruption of oligomeric structure into monomeric structures [33] that have anti-apoptotic properties. Phosphorylated Hsp27 has the ability to suppress cell death signaling through inhibiting caspase-3 activity by binding to pro-caspase-3 [34], inhibiting interaction with cytochrome c [35] or activating upstream protective signaling kinases such as Akt/PKB [36,37].

The function of oligomeric Hsp27

Unlike other HSPs (such as Hsp70), Hsp27 is not regulated by ATP and its protein binding activity depends on the level of oligomerization and phosphorylation of the protein. Although Hsp27 molecular chaperone lacks the ability to refold misfolded protein, it binds to protein insoluble aggregates and lead to enhanced proteasomal degradation [38,39], decrease aggregation of misfolded protens [40] or inhibition of death signaling. Hsp27 promote cell survival by reducing the levels of misfolded protein. In *in vitro* assays, oligomeric Hsp27 is shown to inhibit cap-dependent protein translation by binding to eIF4G, a protein that binds to mRNA guanosine cap and lead mRNA to ribosome for translation [41]. Several studies suggested that Hsp27 is particularly potent neuroprotectant. Although the function of Hsp27 in aggregate-associated neurodegeration is poorly understood, in several studies of Alzheimer's disease (AD) models, Hsp27 bound to Aβ1-40 and decreased aggregation and cytotoxicity in cerebrovascular cultures [42]. It has been shown that Hsp27 expression is increased in AD brain

that has high occurrence of neurofibrillary tangles [43,44]. This may suggest that the expression of Hsp27 is increased to stabilize and diminish toxicity of neuronal aggregates [45].

The molecular structure of Hsp27

α-crystallin domain in C-terminal is essential in forming β-pleated sheets that allow self-assembly of Hsp27 peptides into oligomeric structure. Although the N-terminal contains serines (residue 15, 78 and 82) that are susceptible to phosphorylation for oligomerization regulation [46], N-terminal also consists of highly conserved hydrophobic motif (WDPF) [47,48] that stabilizes the formation of the constitutive oligomeric state of Hsp27 [49,50]. Formation of oligomeric structure requires both C-terminal domain and hydrophobic motif in N-terminal [51]. Most of the studies about the role of Hsp27 in chronic neurodegenerative diseases are correlative and relatively lacking in functional analysis. Oligomeric Hsp27 is particularly interesting as its chaperone activity may help reduce the pathologies of neurodegenerative diseases. We hypothesize that small molecules that binds to Hsp27 *in vitro* and lead to exposure of hydrophobic residues may induce oligomerization of Hsp27. Using turbidity measurement, we screened six preliminary small molecules against monomeric Hsp27 and we discovered that glycyrrhetinic acid can effectively promote aggregation of Hsp27. As glycyrrhetinic acid consists of highly hydrophobic aromatic groups that might induce self-assembly through interaction with Hsp27 hydrophobic motifs, we extended our Hsp27 screening with 29 sterols. These results showed that several sterols contribute to significant increase in Hsp27 self-assembly. Small molecule that can stimulate natural cellular defense through boosting Hsp27 chaperone activity may represent potential therapeutic agents.

MATERIALS AND METHODS

Materials: Amyloid-β (1–42) peptide was purchased from EZBiolab (Westfield, IN). DMSO, HFIP, PBS and DMEMF-12 were purchased from Sigma–Aldrich. Compounds used in the fluorescence screen were synthesized internally [36] or purchased from Sigma–Aldrich, Fluka, Fisher Scientific, and Cayman Chemicals (Ann Arbor, MI). Anti-amyloid b antibody (6E10) was purchased from Calbiochem (San Diego, CA). Fmoc-protected amino acids, biotin, and Wang resin were purchased from Anaspec. Unless otherwise noted, all solvents for peptide synthesis were purchased from Fisher. DIC and HOBt were purchased from Fluka (Milwaukee, WI) and GenScript (Piscataway, NJ), respectively. Microwave-assisted peptide synthesis was performed on a Biotage Initiator EXP using the times and temperatures described. All NMR data were collected and analyzed on a Varian 600 MHz system using VnmrJTM version 2.2 revision C. Mass spectrometry data were obtained on a Micromass LCT time-of-flight mass spectrometer in the ES+ mode. All HPLC runs were performed on a Beckman-Coulter HPLC system measuring at 254nm with a Waters Spherisorb 10μm ODS2 4.6X250mm analytical column at a flow rate of 1 mL/min (acetonitrile:water gradient containing 0.1% TFA) All fluorescence readings were taken on a SpectraMax M5 multi-mode plate reader (Molecular Devices, Sunnyvale, CA).

Amyloid-β stock preparation: One milligram samples of Aβ (1–42) peptide were dissolved in 200 μL hexafluoroisopropanol (HFIP) and aliquoted to obtain 0.1 mg stocks. HFIP was removed under nitrogen to provide a thin film and these stocks were stored at -30 C until ready for use. Immediately prior to the start of each experiment, an aliquot was dissolved in DMSO (see below). Fibrils were obtained by adding phosphate buffered saline (PBS; pH 7.4) to a final amyloid concentration of 25 μM (1% final DMSO concentration). These solutions were vortexed,

sonicated for 75 s, and agitated for 48 h at 37 C. Oligomers were obtained by adding DMEMF12 media (Gibco) to a final amyloid concentration of 25 μM (1% final DMSO concentration), followed by vortexing, sonicating for 75 s, and incubating for 48 h at 4 C without agitation. LMW-Aβ was obtained by adding PBS (pH 7.4) to a final amyloid concentration of 25 lM (1% final DMSO concentration), vortexing, sonicating for 75 s and using these samples immediately.

Transmission electron microscopy: Freshly suspended Fb or aggregated sample (25 μM; 5 μL) was added to glow-discharged, Formvar/carbon 300-mesh copper grids (Electron Microscopy Sciences, Hatfield, PA) and incubated for 1.5 min at room temperature. Excess sample was blotted off with filter paper and each grid was washed twice with ddH2O. Uranylacetate (1%, 3 μL) was added to each grid and incubated for 1 min. Excess sample was blotted off and grids were then dried for 15 min. Samples were visualized on a Phillips CM-100 transmission electron microscope at 80 kV and 94,000Xmagnification.

Thioflavin T fluorescence assay: 100 μL of each compound at different concentrations was added to 9 μL fibrils or oligomers (25 μM) or 9 μL PBS or DMEM-F12 (1% DMSO) in triplicate to a black 96-well plate and incubated for 48 hours. 100 mm aqueous solution of Thioflavin T (ThT) was prepared and filtered through a 0.2 mm filter. The Aβ oligomers and fibrils samples after 48 incubation were diluted to a final concentration of 5 mm into glycine/NaOH (90 mm, pH 8.5) that contained 10 mm ThT. Fluorescence was measured in 96-well plates by using a Fluostar microplate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany) at an excitation wavelength of 446 nm and emission at 490 nm. The background fluorescence is subtracted. The percent Aβ aggregation was calculated with the formula:

$$\%\, aggregation = \frac{F - \overline{F_{ThT}}}{\overline{F_{A\beta}} - \overline{F_{ThT}}}\, X\, 100$$

Where F is the fluorescence reading, $\overline{F_{ThT}}$ is the average intrinsic fluorescence of ThT; $\overline{F_{A\beta}}$ is the average background fluorescence of Aβ aggregate only.

WKLVFF peptide synthesis: Microwave-assisted solid-phase Fmoc-peptide synthesis was performed generally as described [69,70]. Briefly, for each peptide, 1eq of Wang resin (1mmol/g) was activated with 6eq each of DIC and HOBt for 30 minutes stirring in DMF at room temperature, after which 0.6 eq DMAP and 10 eq Fmoc-Phe were added. The reaction mixture was then irradiated for 60 minutes at 60°C (pre-stirring 30 seconds). The coupling efficiency was measured by filtering and washing (DMF, methanol, and DCM) 1-2 mg of resin, to which 3 mL of 20% piperidine in DMF was added in a quartz cuvette. The absorbance at 290 nm (A290) was then measured after a 5 minute incubation and inverting the cuvette several times. The percentage of coupled resin was then calculated using the following equation: % coupled = [(A290) / (mass of resin (mg)]*100%. The coupling efficiency for each peptide was > 70%. The resin was then washed with DMF, methanol, and DCM. The Fmoc group was then removed (20% piperidine in DMF) by irradiating the mixture for 3 minutes at 60 °C, and then resin was again washed as described. The peptides were subsequently built using 3 eq of each Fmoc-protected amino acid, and 5 eq each of DIC and HOBt. The reagents were combined in DMF and, after 5 minutes of stirring, were irradiated for 8-15 minutes at 60 °C, depending upon the amino acid. Coupling efficiency was evaluated using a standard Kaiser test. The resin was washed and the Fmoc group was removed as already described. These steps were also followed for the addition of biotin on the N-terminus of each peptide, except for Fmoc deprotection.

Fluorescence screen: Each compound was dissolved in DMSO to a final concentration of 100 mM and then diluted to 50 μM with ddH2O (1% final DMSO concentration). 100 μL of each

compound was added to 9 μL Fb fibrils or oligomers (25 μM) or 9 μL PBS or DMEM-F12 (1% DMSO) in triplicate to a black 96-well plate and incubated for 10 min. Fluorescence spectra were then recorded at four excitation and emission values: (a) Ex 290 nm, Em 320– 520 nm; (b) Ex 350 nm, Em 380–650 nm; (c) Ex 400 nm, Em430–620 nm; (d) Ex 450, Em 480–650. Background fluorescence of fibrils, oligomers, PBS, and DMEM-F12 in the presence of 100 μL ddH2O only (1% DMSO) was subtracted.

Hsp27 protein production

Protein expression: An N-terminally hexahistidine tagged human heat shock protein 27 (His6Hsp27) was expressed in BL21 cells using a pET 28b vector. Cultures of transformed cells were grown to an optical density at 600nm of 0.8AU in Luria broth media supplemented with 50μg/mL kanamycin at 37C. Cultures were induced with 500μM IPTG and incubated at 30C for 8-16 hours.

***Protein purification*:** After incubation, cells were harvested by centrifugation at 4000rpm for 15 minutes and resuspended in 1/25 the culture volume of lysis buffer (20mM Tris pH8.0, 100mM NaCl, 6M urea, 5mM β-mercaptoethanol, 15mM imidizole) and Protease Inhibitor Cocktail Tablets (Sigma-Aldrich). The suspension was lysed by sonication on ice until it was non-viscous and the insoluble debris were removed by centrifugation at 4000rpm for 20 minutes. The supernatant was then loaded onto Ni-NTA resin (3ml per liter culture volume) pre-equilibrated with lysis buffer and washed with 10 column volumes of wash buffer (20mM Tris pH8.0, 100mM NaCl, 6M urea, 5mM β-mercaptoethanol, 30mM imidizole). 10 half-column volumes of elution buffer (20mM Tris pH8.0, 100mM NaCl, 6M urea, 5mM β-mercaptoethanol, 150mM

imidizole) were used to elute the protein. Fractions containing protein were pooled and concentrated to about 20mg/mL using a 10kDa MWCO Amicon centrifugal filter unit.

Protein refolding: 1ml of the concentrated eluate from the Ni-NTA column was injected onto a Superdex 200 HR 10/30 column equilibrated with folding buffer (20mM NaH_2PO_4/ Na_2HPO_4 pH 7.2, 100mM NaCl) at room temperature. The refolded protein peak was collected and concentrated to about 10mg/ml before being flash frozen and stored at -80C.

Turbidity assay: 10µL of 10X compounds in 10% DMSO and 90µL 1X PBS and control buffer (10%DMSO and 90µL 1X PBS) were prepared. Stocks and buffer were dispensed into 96-well clear bottom plates. The absorbance spectrum of small molecules only was calibrated as background. 25µM of Hsp27 is added. Absorbance was monitored at 300nm for every 15 seconds using SpectraMax M5 multi-mode plate reader. Temperature was set at room temperature. The absorbance was read for 60 minutes. The end point reading is taken and percentage change of absorbance is calculated.

RESULTS

Preparation and characterization of Aβ(1-42) fibrils and oligomers

Since we are interested in investigating whether small molecules and short fragment peptide have distinct interactions with Aβ fibrils and oligomers, we adopt known conditions to induce Aβ monomer to generate relatively homogenous Aβ fibrils and oligomers *in vitro* [52-54]. As illustrated in Figure 1 and explained in detail in Material and Methods, Aβ(1-42) oligomers were prepared by 48-hour incubation in DMEM -F12 at 4℃ without agitation, while Aβ(1-42) fibrils were prepared by 48-hour incubation in PBS at 37℃ with agitation. To confirm homogeneity of each Aβ fibrils and oligomers samples, the samples were collected at 0, 8, 16 and 24 hour time points to be analyzed by transmission electron microscopy (TEM) (Figure 2). The TEM showed that the oligomer samples were characterized by predominantly spherical structures and free of linear fibrils. These fabricated oligomers share similar structure and neurotoxicity properties with the soluble Aβ samples from AD patients. The amount of Aβ aggregates in each homogeneous Aβ conformation samples was found to be increased over the duration of 24 hours.

Thioflavin T fluorescence screen for Aβ conformation-specific small molecules

The ability of small molecules to interact with specific Aβ conformations was examined by assessing the amount of Aβ aggregates developed in each samples over time using Thioflavin T (ThT) fluorescence assay. ThT is a common fluorescent probe that could be used to quantify amyloid aggregates. ThT does not bind to Aβ(1-42) monomers. However, ThT can attach to binding sites that are formed to upon Aβ self-assembly and ThT exhibits enhanced fluorescence (Figure 3). More Aβ aggregates correspond to higher ThT fluorescence. A collection of 24 small

molecules that were previously reported as Aβ ligands or Aβ self-assembly inhibitor was chosen for screening. The small molecules library includes diverse classes of chemical scaffolds, such as congo-red-like class, azo aryls, thiazines, tetracyclines and monophenyls. We aimed to screen these small molecules with ThT to identify the compounds that could recognize distinct molecular structure that differentiates Aβ fibrils from oligomers and selectively inhibit fibrils or oligomers. All small molecules that were screened in this assay do not have intrinsic fluorescence or interaction with ThT that will affect the intensity of ThT fluorescence to avoid false positive. Small molecules with different concentrations were added to 25μM oligomers and 25μM fibrils samples at 0-hour (the moment Aβ monomers are first incubated in different growing conditions), 16-hour and 24-hour time points. After 48-hour incubation, we screened for ThT fluorescence changes at Ex 446nm and Em 490nm in these samples (Figure 4). Most of the compounds inhibit aggregation in both Aβ fibril and oligomers. However, we discovered that the fluorescence ThT was selectively quenched in the presence of five compounds (no. 13-15, 18, 22) with either fibrils or oligomers as the compound concentrations increase; Three of these compounds (no. 13-15) were from tetracycline class that significantly quenched fluorescence of ThT after interacting with only Aβ fibrils, but not oligomers, at all time points. Increase in ThT fluorescence suggests that the compound may induce Aβ aggregation, while decrease in ThT fluorescence suggests that the compound may inhibit Aβ aggregation. This result is not only consistent with previous finding that tetracycline can inhibit self-assembly of prion amyloid fibril [23], but also suggests that tetracyclines can selectively recognize molecular structure is uniquely available in fibrils but not in oligomers.

Tetracyclines selectively inhibit Aβ fibril aggregation, but not oligomer self-assembly

We compare the percent aggregation of fibrils and oligomers at the highest concentration of the tetracyclines (Figure 5A) to of circumin. Circumin is a known Aβ ligand that inhibits aggregation of both fibrils and oligomers [55] and hence functions as a positive control for aggregation inhibition. The percent aggregation of fibril with the highest concentrations of the tetracyclines was low and was comparable to the percent aggregation of fibril with the highest concentration of circumin at all time points (Figure 5B). However, the percent aggregation of oligomers with the highest concentrations of the tetracycline and tetracycline derivatives was significantly higher than the percent aggregation of oligomer with the highest concentration of circumin, and there was an increasing trend for rolitetracycline across time points (Figure 5C). This shows that tetracyclines inhibit Aβ self-assembly into fibrils at all time points but fail to inhibit oligomerization. The result also may suggest that rolitetracycline is able to inhibit aggregation of LMW oligomers in earlier time points but gradually lose this ability as the mature oligomer is formed at later time points. We also compare the effectivenss of tetracyclines in inhibiting fibril aggregation by comparing the values of $1/IC_{50}$ (Figure 5D). The analysis shows that tetracycline is the most effective inhibitor in all time points. Minotetracycline is more effective than rolitetracycline at 0 time point but is less effective than rolitetracycline at later time points. By analyzing the chemical structure of tetracyclines, we may deduce that the hydrophobic groups on tetracycline may play a major role in binding to critical hydrophobic residues on Aβ, such as KLVFF motif, and that the tetracycline binding site is masked when Aβ is packed into globular oligomers.

Fluorescence of WKLVFF peptide is quenched by the presence of Aβ fibrils but not oligomers

Previous studies has shown that KLVFF short pentapeptide fragment inhibit Aβ fibrillization by binding to the KLVFF hydrophobic core on full-length Aβ peptide [25] (Figure 6A). To investigate whether KLVFF peptide has distinct interaction with different conformations of Aβ aggregates, we manipulated this synthetic pentapeptide to explore the configuration and location of hydrophobic core in oligomers and fibrils. Tryptophan (W) was added to the N-terminal of the KLVFF that was manufactured by solid-phase protein synthesis (Figure 6B). The indole group on tryptophan fluorescents at optimized wavelengths of Ex280 and Em350, therefore the peptide could be screened with Aβ oligomers and fibrils using fluorescence assay. WKLVFF was incubated with 25μM preformed LMW aggregates oligomers or fibrils samples for 10 minutes before recording its fluorescence. We observed 55.44±4.08% quench in WKLVFF fluorescence with the presence of Aβ fibril, 29.81±11.75% quench fluorescence with the presence of LMW aggregates, but 8.40±3.13% increase in fluorescence with the presence of oligomers (Figure 6C,D). The results may propose that the WKLVFF binds to the hydrophobic core on Aβ fibrils and LMW aggregates, and thus quenches its fluorescence, but fail to bind to Aβ oligomers. Hence, this also indicates that Aβ fibrils and LMW aggregates share similar configuration and packing of hydrophobic core compared to oligomers. WKLVFF may also binds at the growing end of the Aβ aggregates, which binding sites are not available in mature spherical oligomers. Moreover, preliminary study has ruled out the possibility of WKLVFF to self-assemble *in vitro* as the percent fluorescence quench of WKLVFF that are suspended in buffer for 13 days when interacting with Aβ fibrils is comparable to the percent fluorescence quench of freshly prepared WKLVFF. This result is coherent with precedent literature that KLVFF pentapeptide does not self-assemble [56].

WKLVFF binds to the same hydrophobic core as KLVFF pentapeptide

To test whether the binding site of WKLVFF differs from KLVFF upon the modification that we made to enable fluorescence assay, we performed WKLVFF and KLVFF competition assay with Aβ fibrils. The results showed that there was no fluorescence quenching of WKLVFF observable in the competition assay (Figure 7), which suggested that WKLVFF and KLVFF compete for the same binding sites on Aβ fibrils in all concentrations of WKLVFF.

WKLVFF- Aβ fibril interaction causes blue shift in fluorescence spectra

When we optimize the excitation and emission wavelengths of WKLVFF for the fluorescence screen at Ex280 and Em335-400, we discovered that the fluorescence spectrum of WKLVFF when interacted with Aβ fibrils exhibits 12nm blue shift, but does not exhibit any changes when interacted with Aβ oligomers. The blue shift suggested that positive charge, possibly from Lys residue was exposed in fibrils [29].

Kinetics of Hsp27-small molecules interaction

We measured the kinetics of Hsp27 aggregation through turbidity measurement in the presence of different small molecules to identify the compound that binds to Hsp27 *in vitro* and leads to exposure of hydrophobic residues that subsequently induces oligomerization of Hsp27. Monomeric Hsp27 independent of small molecules cannot aggregate *in vitro* within 1 hour (Figure 9A). We screened six preliminary small molecules against monomeric Hsp27 and we discovered that glycyrrhetinic acid can effectively promote aggregation of Hsp27. Turbidity assay showed that Hsp27 aggregation increased rapidly within the first 30 minutes upon interaction with glycyrrhetinic acid. The result also showed that 6-ketocholestanol and ursodiol

could increase oligomerization of Hsp27 but no lag phase of oligomerization kinetics was observed in the presence of these two small molecules (Figure 9B). Other small molecules, including terbutaline hemisulfate, anisomycin and captopril, did not increase the turbidity measurement of monomeric Hsp27, hence suggesting that these small molecules could not induce oligomerization of Hsp27 (Figure 9B).

Glycyrrhetinic acid, 6-ketocholestanol and ursodiol could induce Hsp27 oligomerization

Since glycyrrhetinic acid, 6-ketocholestanol and ursodiol could promote Hsp27 oligomerization, we screened these compounds at different concentrations from 10nM to 100μM with 25μM monomeric Hsp27 to identify the minimum concentration these compounds that could induce oligomerization. The results reported that the 500nM is the minimum concentration for glycyrrhetinic acid and 6-ketocholestanol to promote Hsp27 self-assembly is 500nM, while ursodiol only showed its effectiveness at relatively higher concentration at 1μM. We also compared the effectiveness of these compounds in inducing Hsp27 oligomerization with anisomycin as negative control. According to the analysis of percent change in absorbance in turbidity measurement, glycyrrhetinic acid is the most potent Hsp27 oligomerization inducer, followed by 6-ketocholestanol and ursodiol (Figure 10).

Screen for Hsp27 oligomerization-inducing sterols

Glycyrrhetinic acid, 6-ketocholestanol and ursodiol may differentiate themselves from other small molecules that do not induce Hsp27 self-assembly by having highly hydrophobic groups in their chemical structures. These hydrophobic groups may promote Hsp27 oligomerization through interaction with Hsp27 hydrophobic motifs. As these compounds share similar structure

with sterols, we extended our turbidity assay to further explore changes in aggregation of 25μM Hsp27 monomers with the presence of 29 sterols at 100μM concentration (Figure 11).

We discovered that 15 out of 29 sterols could significantly induce Hsp27 oligomerization (>50% in absorbance). By analyzing the difference between the percent change in absorbance at 0hr and 1hr, 5a-cholestan-3b-ol-6-one, 4-cholesten-3-one and estrone have notably increased the absorbance greater than 100% (Figure 12), therefore are most potent in inducing Hsp27 aggregation. These results showed that sterols could contribute to significant increase in Hsp27 self-assembly and may be further manipulated to develop potential therapeutic agents that enhance Hsp27 chaperone activity.

DISCUSSION

Recent findings have shown that Aβ 1-42 can self-assemble into different conformations, which some conformations such as annular oligomers are more neurotoxic than fibrils. Our goal is to determine substances, including drugs and biologics that could bind and differentiate between fibrils and oligomer, or specifically impact the formation of distinct Aβ conformations. The substance that could differentiate the distinct molecular structures on fibrils and oligomers could be developed as specific probes that is more superior than Bis-ANS and Congo Red, which lack such property [67,21]. We employed two approaches: screening homogenous Aβ fibrils and oligomers samples that were prepared artificially with 24 small molecules using Thioflavin T fluorescence assay, and with modified KLVFF short pentapeptide with fluorescence assay.

Tetracycline may bind at hydrophobic core in Aβ peptide growing end that is essential for self-assembly.

In the first approach, we monitored the fibril or oligomer formation with the presence of small molecules using ThT fluorescence assay. Through the screen, we discovered that three tetracyclines that are capable of specifically inhibiting fibril formation at all time points. In the time dependent study, ThT fluorescence assay reported that tetracyclines lost their ability to bind to Aβ oligomers and inhibit aggregation when tetracyclines were added later in time. We propose a model that tetracyclines inhibit Aβ peptide self-assembly by binding to the growing end of Aβ (Figure 13). The tetracycline binding site at the growing end is available at all time points in elongated fibrils, but the binding site may be hidden once mature oligomer is formed. Moreover,

since tetracyclines, especially rolitetracycline can inhibit aggregation of both oligomer and fibril when it is added to Aβ monomers, these compounds could be used as therapeutics that inhibit the formation of neurotoxic oligomer.

Previous findings showed that tetracyclines have anti-amyloidogenic activity with Aβ1-42 peptides [61], besides that they interact with hydrophobic side chains in prion proteins and inhibit formation of amyloid fibrils [23]. However, the molecular basis of tetracyclines anti-amyloidogenic properties is poorly understood. From our findings, we deduced that the extended hydrophobic core formed by aromatic moieties in tetracyclines allow stable hydrophobic interaction with the hydrophobic or aromatic residues on Aβ that are crucial for self-assembly. Therefore, tetracyclines are not only pharmacologically interesting, but also have selective inhibition properties that allow us to investigate the mechanism of Aβ self-assembly by identifying the distinct molecular structure that differentiates Aβ oligomer from fibrils.

KLVFF hydrophobic core exposure pattern may be distinctive in Aβ oligomers and fibrils

In the second approach, we observed the change in fluorescence of modified pentapeptide WKLVFF upon interaction with LMW aggregates, pre-formed fibrils and oligomers samples. Through the screen, we observed quench in WKLVFF fluorescence only occurs with the presence of fibrils or LMW aggregates, but not oligomers. This suggested that LMW aggregates and fibrils share similar conformation and have similar packing of KLVFF on the Aβ peptide. Moreover, we also discovered a significant blue shift when WKLVFF specifically interacted with fibrils. The blue shift and WKLVFF fluorescence quenching could be employed as probes

that permit selective recognition of different Aβ isoforms. Since fluorescence of WKLVFF only quenched when it interacted with LMW aggregates and fibrils, but not with oligomers, it is possible that the WKLVFF binds to KLVFF residues on Aβ peptide as a growing end binder and KLVFF residues may be partly or entirely buried in pre-formed oligomer (Figure 14). This coincides with the findings in small molecules screening and by correlation, tetracycline may have similar mechanism in interacting with different Aβ conformations and bind to the similar essential hydrophobic core as WKLVFF. Further work in needed to test this hypothesis.

Role of π –stacking in Aβ peptide self-assembly

π-stacking is important in creating a stable intermolecular interactions from stacked arrangement between aromatic molecules. The aromatic groups in KLVFF hydrophobic core may interact with aromatic groups on tetracyclines and WKLVFF peptide. Nowick J.S. (2008) suggested that hydrogen bonds alone are not sufficient in initiating Aβ peptide self-aggregation and that hydrophobic interaction generated by phenylalanine groups is fundamental in stabilizing the Aβ aggregation [1]. π-stacking can allow stable formation of supramolecular structure as it is thermodynamically favorable and it can form highly ordered structure as it adopts specific directionality and orientation [57]. Previous studies have shown that FF motif in various truncated Aβ peptides can inhibit Aβ peptide self-assembly [58-60]. It was also observed that Congo red interacted with Aβ peptides through aromatic group that serves as its major functional group [34]. All these results, together with our findings, supported that the π –stacking in Aβ peptide hydrophobic core (KLVFF) could greatly impact the ability to self-assemble into different conformations. Our results further suggest that the hydrophobic core may be exposed

distinctly in oligomers and fibrils. The understanding of this structure allows further rational design of small molecules or biologics that could selectively inhibit or to probe for specific conformations of Aβ.

Hydrophobic and/or aromatic small molecules effectively induce Hsp27 oligomerization

Previous studies have shown that Hsp27 has multiple cellular functions depending on whether it is in oligomeric structure or monomeric structure. Just like Aβ peptide, Hsp27 relies on hydrophobic domains to initiate and stabilize oligomerization. Essential hydrophobic interactions are established by the α-crystallin domain in C-terminal that permits formation of β-pleated sheets and the highly conserved hydrophobic motif (WDPF) in N-terminal [47-48]. In our study, we wish to detect the small molecules that could modulate surface hydrophobicity of Hsp27 peptide. We hypothesized that small molecule modulators that binds to Hsp27 will increase its exposure of hydrophobic surfaces and hence leads to increase in oligomerization. Towards this goal, we monitor the change in absorbance in turbidity assay when Hsp27 interacts with small molecules and we discovered that small molecules with high hydrophobicity and aromatic groups, such as glycyrrhetinic acid and several sterols, could induce Hsp27 self-assembly into oligomeric chaperones in vitro.

Based on our results, we deduced that Hsp27 self-assembly, just like Aβ peptide aggregation, is initiated and stabilized by on hydrophobic interactions or π-stackings. This could be proved by assessing the percent changes in absorbance induced by small molecules of similar structures but have different functional groups (Figure 15). For instance, 4-cholesten-3-one could enhance

Hsp27 self-aggregation about three times more than 5-cholesten-3b-ol; estrone significantly increased Hsp27 aggregation but b-estradiol showed negative change in absorbance value. This suggests that the replacement of ketone group with hydroxy group will greatly reduce the ability of sterol in inducing Hsp27 oligomerization. Since ketone is more hydrophobic than hydroxyl group, the result may suggest that the reduction of hydrophobicity of sterol will reduce its ability to bind and interact with Hsp27. These data also suggest that Hsp27 is amenable to drug treatment and reacts by increasing its surface hydrophobicity. Previous findings showed that oligomeric Hsp27 has chaperone activity that is neuroprotective and it can reduce neuronal aggregates. Hence, these sterols that could increase oligomerization of Hsp27 may represent potential therapeutic agents in boosting Hsp27 chaperone activity.

CONCLUSION AND FUTURE DIRECTIONS

These results from fluorescence screen for Aβ conformation-specific small molecules suggest that it is possible to selectively inhibit either Aβ1-42 oligomerization or fibrillization. Besides that, tetracyclines recognize a domain that marks the molecular difference between mature oligomer and elongated fibril.

We can further investigate the binding affinity of tetracyclines for both oligomers and fibrils, and to investigate if tetracyclines bind to KLVFF hydrophobic core by using surface plasmon resonance (SPR). Moreover, tetracyclines, which intrinsically have good bioavailability, could be developed as therapeutics that inhibit fibril formation or as prophylactic that inhibit Aβ monomer from progressing into oligomers or fibrils. However, tetracyclines need to cross blood brain barrier to effectively inhibit Aβ species. Minocycline has the ability to cross blood brain barrier, hence could be a good candidate for in vivo studies.

Previous studies suggest KLVFF residues are important for development of β-pleated sheet on Aβ, which allows Aβ peptide self-aggregation into higher ordered structures. Our research investigated the difference in location and exposure of this hydrophobic core in different Aβ conformations. We discovered that blue shift and WKLVFF fluorescence quenching that are observable could permit selective recognition of fibrils and oligomers. KLVFF could be potential candidate in designing biologics that inhibits Aβ peptide aggregation. However, KLVFF is a peptide and it could not cross the blood brain barrier. This could be solved by the monoclonal antibody against Aβ that can draw soluble Aβ out from the brain into the blood stream [63]. KLVFF derivatives could be injected into AD patients with the antibody so that KLVFF can bind and stop the self-assembly of soluble LMW aggregate species into oligomers or fibrils.

Compared with tetracyclines and other aromatic drugs that might lack specificity in inhibiting Aβ self-assembly, KLVFF, emerged as the 'molecular recognition elements' in Aβ, is a promising inhibitor due to its higher affinity and specificity to the Aβ peptide hydrophobic core.

Modifications can be made to KLVFF to increase its affinity to Aβ peptide, and hence, to increase its inhibition potency. One of the recent study by Reinke A.A. et. al .added linkers of various length in between two KLVFF dimers to enhance the binding affinity and inhibition ability [63]. In that study, it is shown that the longer linkers (~19 to 24 Å) could be used as probes for early LMW Aβ aggregates such as trimers and tetramers, which also have significant neurotoxicity.

Hsp27 has neuroprotectant chaperonic activity when it is oligomerized. Based on the understanding of molecular recognition structure in Aβ peptide that is essential in self-assembly, we aimed to identify small molecules that could increase self-assembly of Hsp27 by increasing its surface hydrophobicity. We discovered that sterols and highly hydrophobic small molecules could achieve such effect. These sterols could be potential drugs that enhance Hsp27 chaperonic properties, as suggested in previous studies that transgenic overexpression or transduction delivery of Hsp27 provided robust neuroprotection. However, overexpression of Hsp27 has been correlated with several human gliomas [64,65] and may contribute to cancer development[66]. Therefore, the small molecules that could increase Hsp27 oligomerization have to be chemically modified to increase specificity to function as a treatment against neurological injury. We currently proceed with larger small molecule library screening with Hsp27 using high throughput thermal denaturation assays to identify more small molecules that could modulate Hsp27 activity.

FIGURES

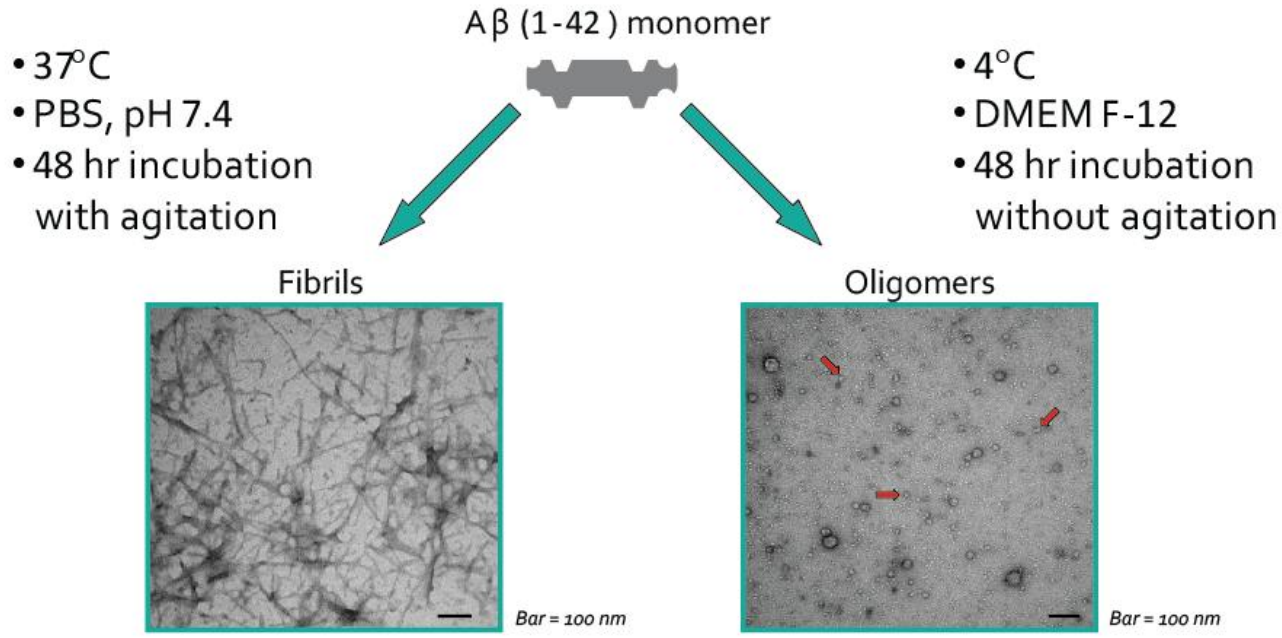


FIGURE 1. Preparation of Aβ(1-42) fibrils and oligomers *in vitro*. Aβ (1-42) monomer can be promoted to progress into distinct homogeneous fibrils and oligomers specieses by manipulating the conditions such as temperature, time, salinity and pH.

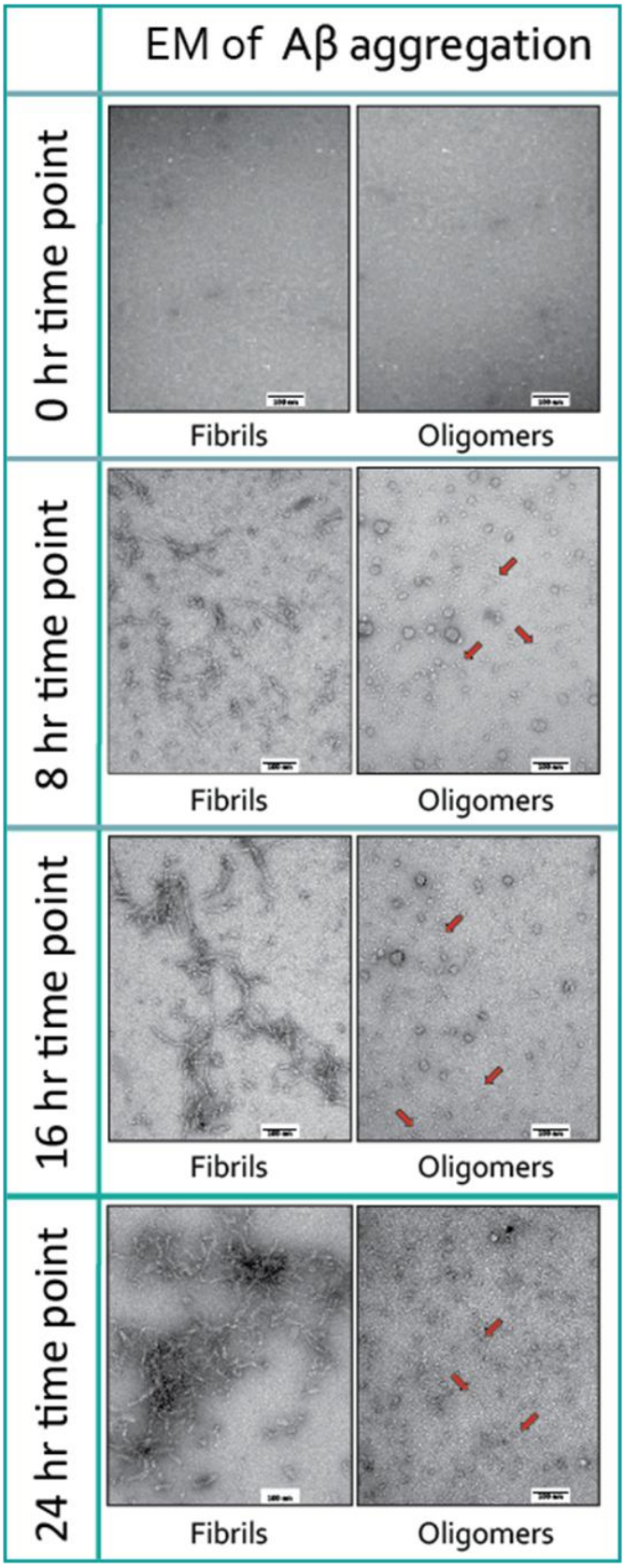
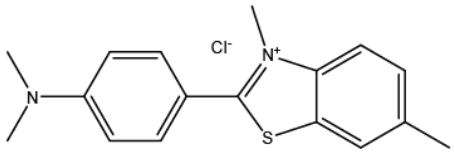
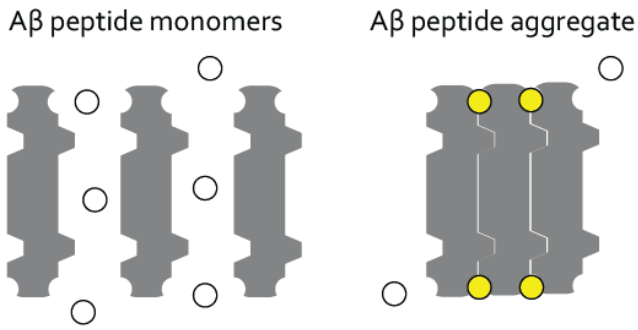


FIGURE 2. Characterization of Aβ(1-42) fibrils and oligomers at different time points by transmission electron microscopy TEM. Artificially induced Aβ (1-42) fibrils and oligomers have distinct ultrastructures and the amount of amyloid aggregates of each conformation increases over the duration of 24 hours.

(A)



(B)



(C)

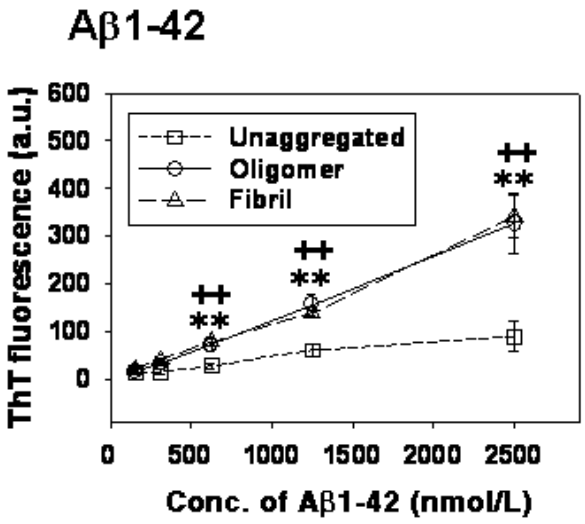


FIGURE 3. Thioflavin T as the fluorescent probe that quantifies Aβ aggregates. (A) Chemical structure of Thioflavin T. (B) ThT binding site is absent in Aβ peptide monomers. Upon Aβ self-assembly, ThT binds to the Aβ peptide aggregates and exhibits enhanced fluorescence. (C) Figure adopted from Figure 3 in Maezawa *et. al.*[21] that shows ThT fluorescence increases as more aggregates are formed in oligomers and fibrils samples. ThT lack the ability to distinguish the intermediate oligomers and maturely developed amyloid fibrils.

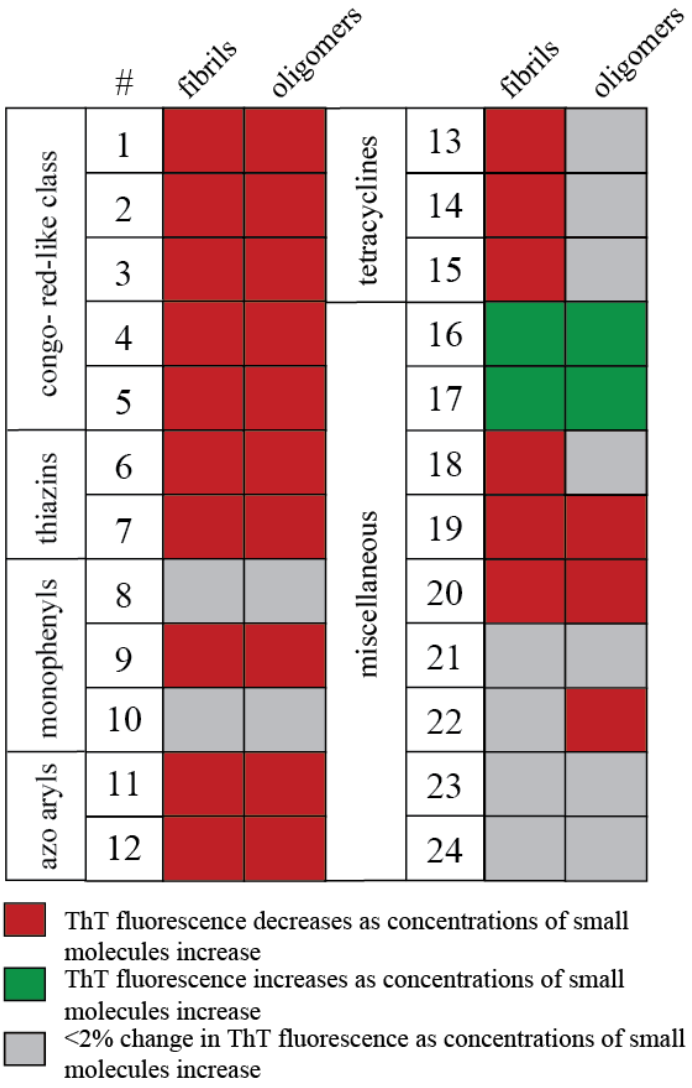
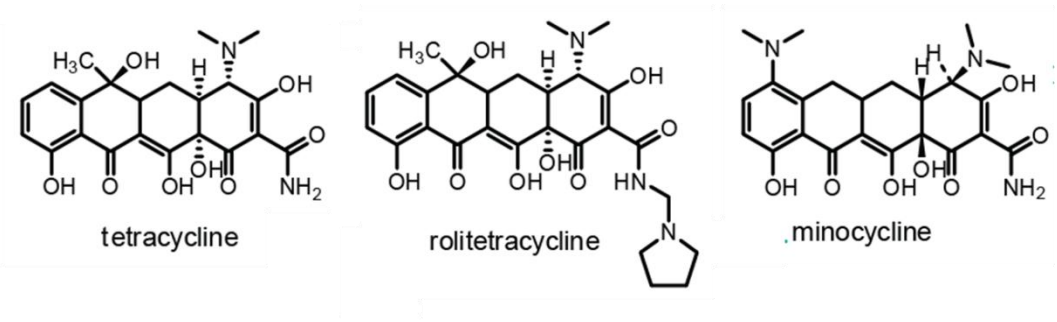
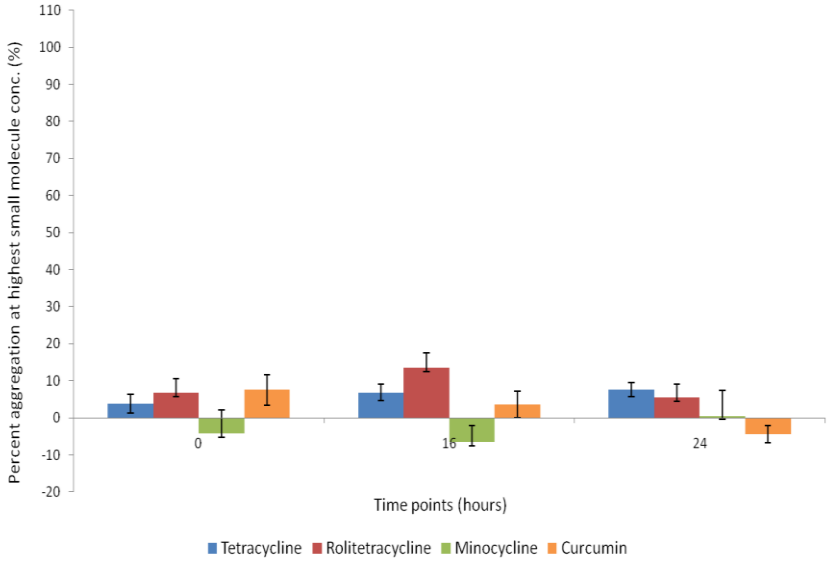


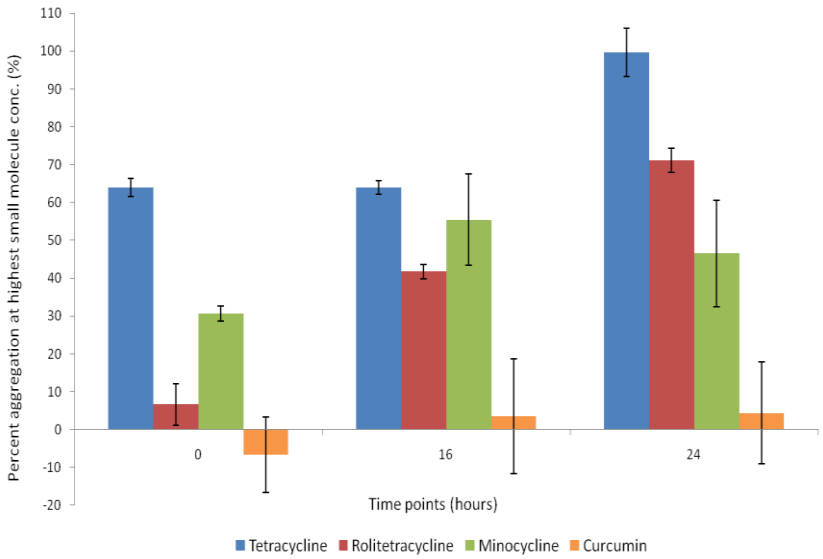
FIGURE 4. Thioflavin T fluorescence screen for Aβ conformation-specific small molecules. (Supp. Fig. 1) The ThT fluorescence was recorded at Ex 446nm and Em 490nm after 25μM oligomers or fibrils samples are incubated with different concentrations of small molecules. The results shown above can be observed regardless of the Aβ incubation time before compounds are added. Based on the analysis of percent aggregation, small molecules can be catagorized into compounds that causes minimal change (<2%) change in ThT fluorescence (grey box), that causes increases ThT fluorescence (green box) and that decreases ThT fluoresncence (red box). Increase in ThT fluorescence suggests that the compound may induce Aβ aggregation, while decrease in ThT fluorescence suggests that the compound may inhibit Aβ aggregation. The tetracyclines all show the ability to inhibit aggregation of Aβ fibril, but not of oligomer.


tetracycline
rolitetracycline
minocycline

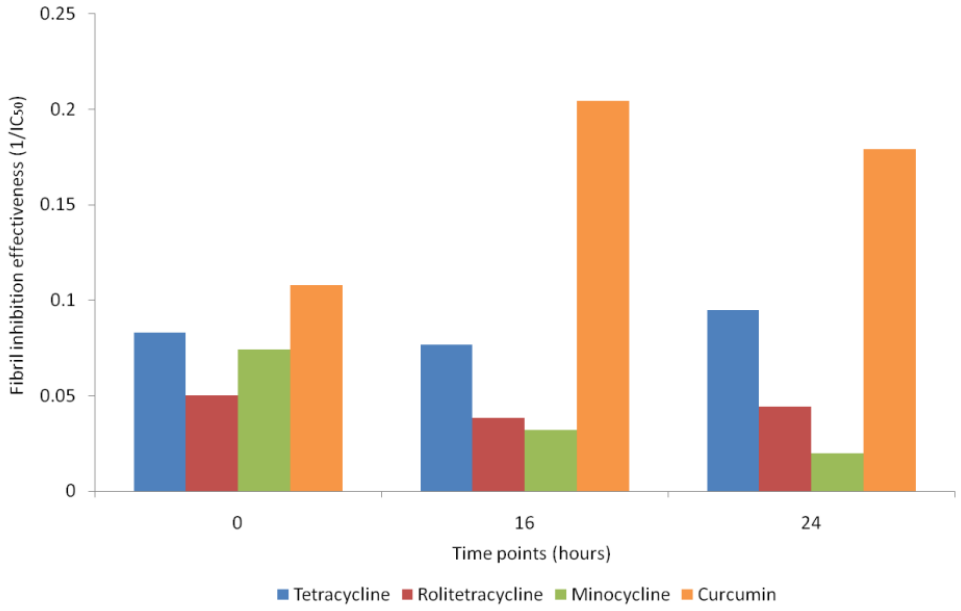
(A)


Percent aggregation at highest small molecule conc. (%)
Time points (hours)
Tetracycline
Rolitetracycline
Minocycline
Curcumin

(B)


Percent aggregation at highest small molecule conc. (%)
Time points (hours)
Tetracycline
Rolitetracycline
Minocycline
Curcumin

(C)



(D)

FIGURE 5. Aβ fibril self-assembly is selectively inhibited by tetracyclines. (A) Chemical structure of tetracyclines used in screening. (B) The percent aggregation of fibril with the highest concentrations of the tetracyclines is low and comparable to the percent aggregation of fibril with the highest concentration of circumin at all time points. (C) The percent aggregation of oligomers with the highest concentrations of the tetracyclines is significantly higher than the percent aggregation of oligomer with the highest concentration of circumin, and there is an increasing trend for rolitetracycline across time points. The result also may suggest that rolitetracycline is able to inhibit aggregation of LMW oligomers in earlier time points but gradually lose this ability as the mature oligomer is formed at later time points. (D) The effectivenss of tetracyclines in inhibiting fibril aggregation is evaluated by $1/IC_{50}$. Tetracycline is the most effective inhibitor in all time points. Minotetracycline is more effective than rolitetracycline at 0 time point but is less effective than rolitetracycline at later time points.

(A)

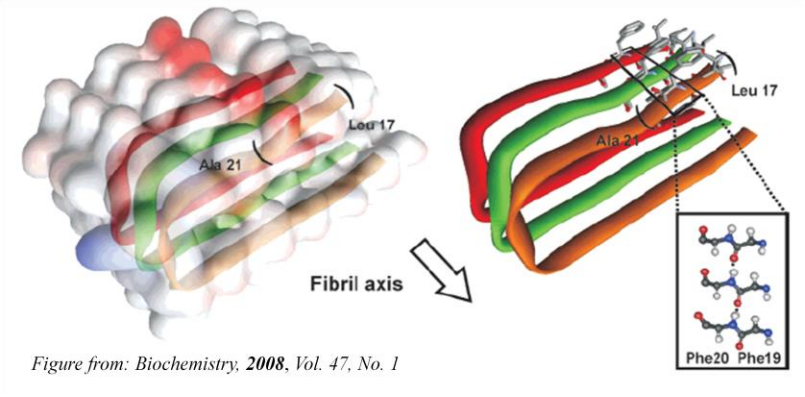
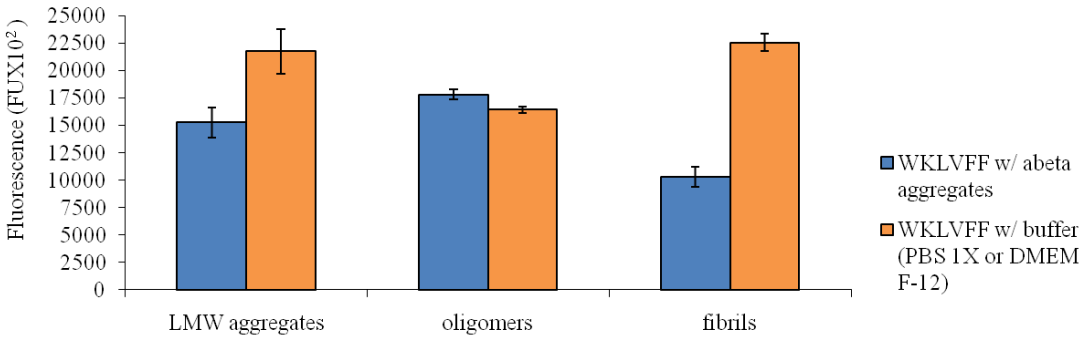


*Figure from: Biochemistry, **2008**, Vol. 47, No. 1*

(B)

(C)



(D)

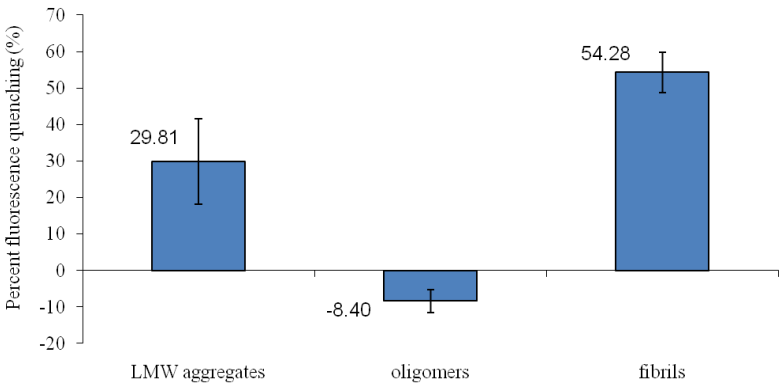
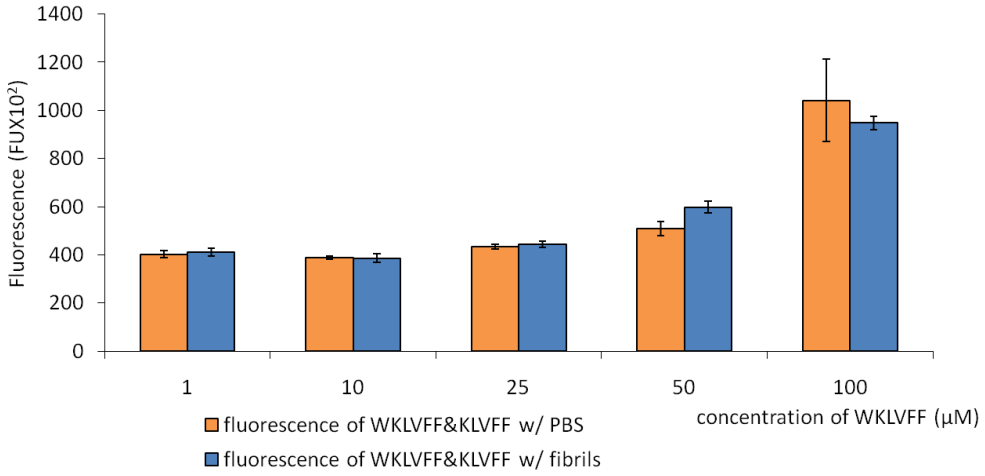
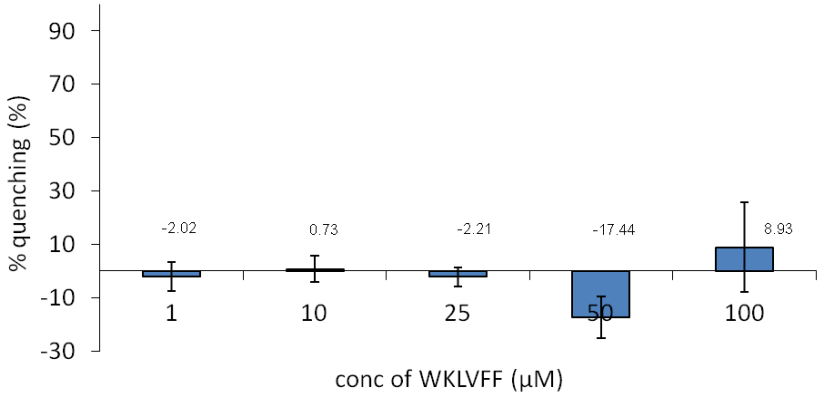


FIGURE 6. Fluorescence of WKLVFF is selectively quenched by interaction with Aβ fibrils and LMW aggregates, but not oligomers. (A) KLVFF binds to homologous sequence in full length Aβ peptide [17]. (B) Chemical structure of WKLVFF used in screening. (C) Interaction between WKLVFF and Aβ fibrils or LMW aggregates, but not oligomers, quench the fluorescence of WKLVFF peptide. (D) The analysis of percent quenching showed that Aβ fibrils quench the fluorescence of WKLVFF about twice as much compared to LMW aggregates.



(A)



(B)

FIGURE 7. KLVFF and WKLVFF competition assay. (A) No fluorescence quenching of WKLVFF was observable at increasing concentration of WKLVFF (B) The analysis of percent quenching showed that Aβ fibrils could not quench the fluorescence of WKLVFF as effectively due to competition of binding site with KLVFF.

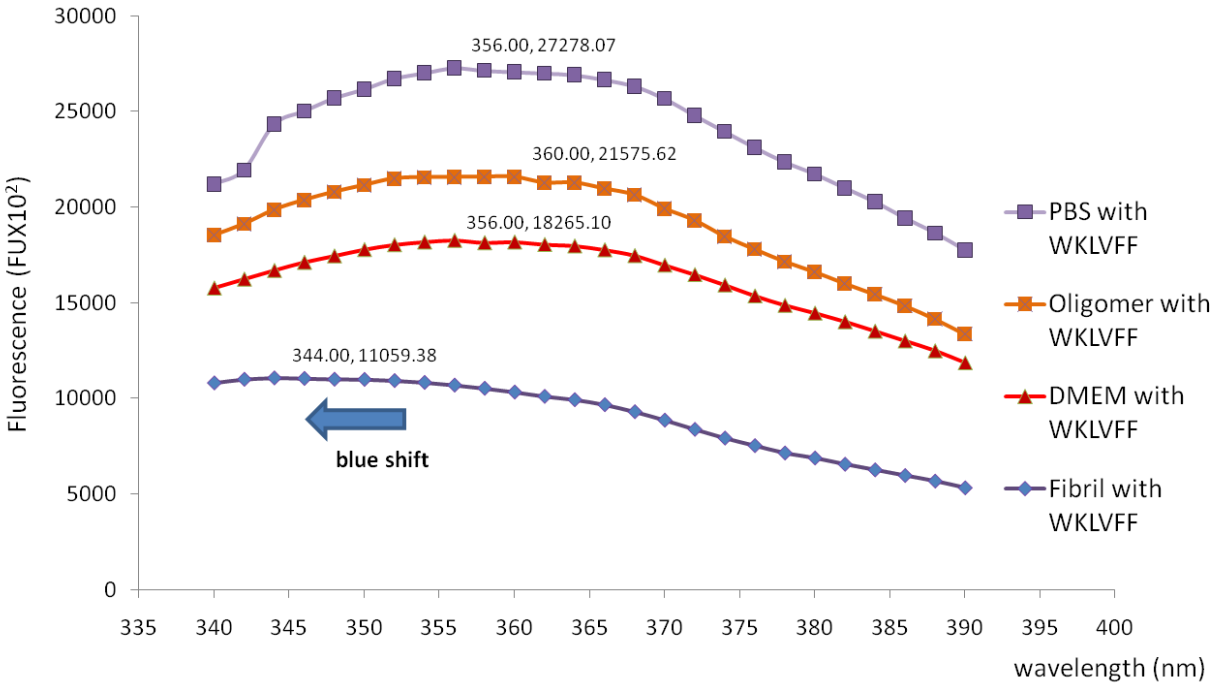
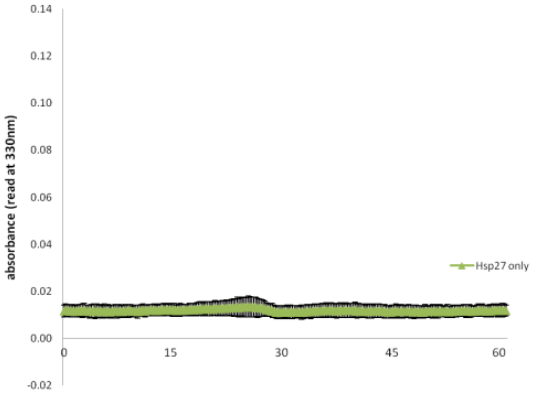
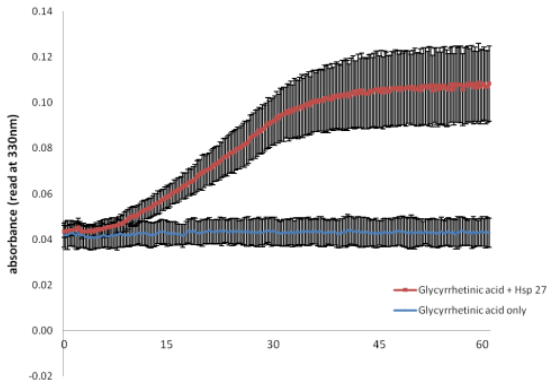


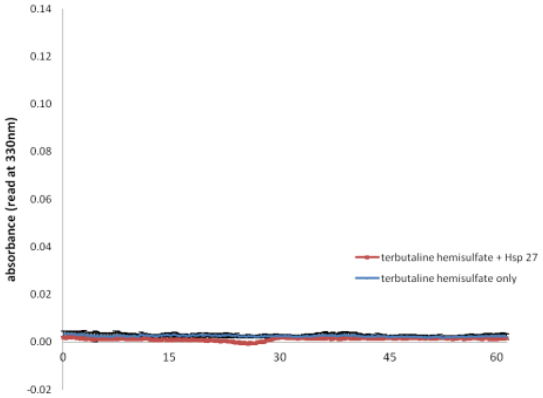
FIGURE 8. WKLVFF-fibril interaction causes blue shift in fluorescence spectrum. When screened at Ex280 and Em335-400, fluorescence spectrum of WKLVFF reported 12nm blue shift when interacted with fibrils, but not with oligomers.

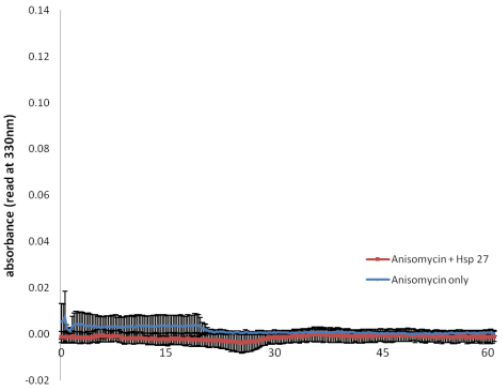
(A)

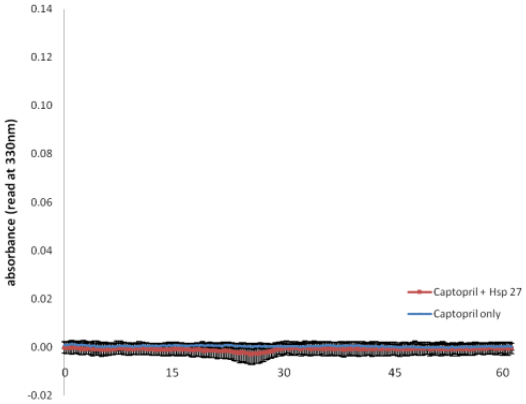

absorbance (read at 330nm)
Hsp27 only

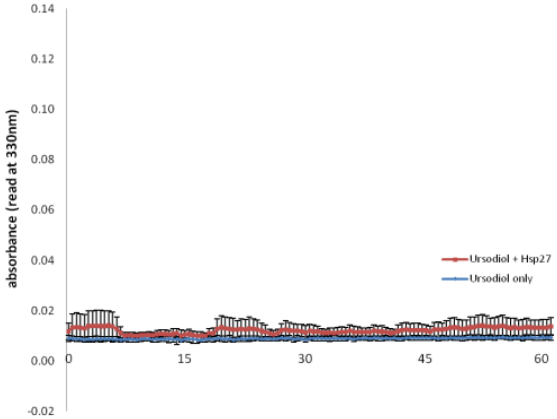
(B)


absorbance (read at 330nm)
Glycyrrhetinic acid + Hsp 27
Glycyrrhetinic acid only


absorbance (read at 330nm)
terbutaline hemisulfate + Hsp 27
terbutaline hemisulfate only


absorbance (read at 330nm)
Anisomycin + Hsp 27
Anisomycin only


absorbance (read at 330nm)
Captopril + Hsp 27
Captopril only


absorbance (read at 330nm)
Ursodiol + Hsp27
Ursodiol only

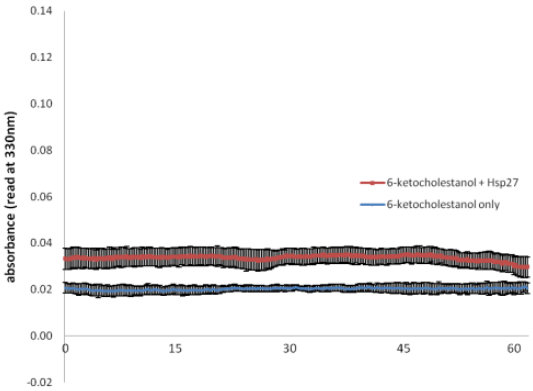

absorbance (read at 330nm)
6-ketocholestanol + Hsp27
6-ketocholestanol only

FIGURE 9. Kinetics of Hsp27-small molecules interaction. (A) Monomeric Hsp27 without the presence of small molecules cannot undergo self-assembly *in vitro* within 1 hour (B) Kinetics of Hsp27 aggregation (25μM) with the presence of 100μM glycyrrhetinic acid, terbutaline hemisulfate, anisomycin, captopril, ursodiol and 6-ketocholestanol was read at 300nm.

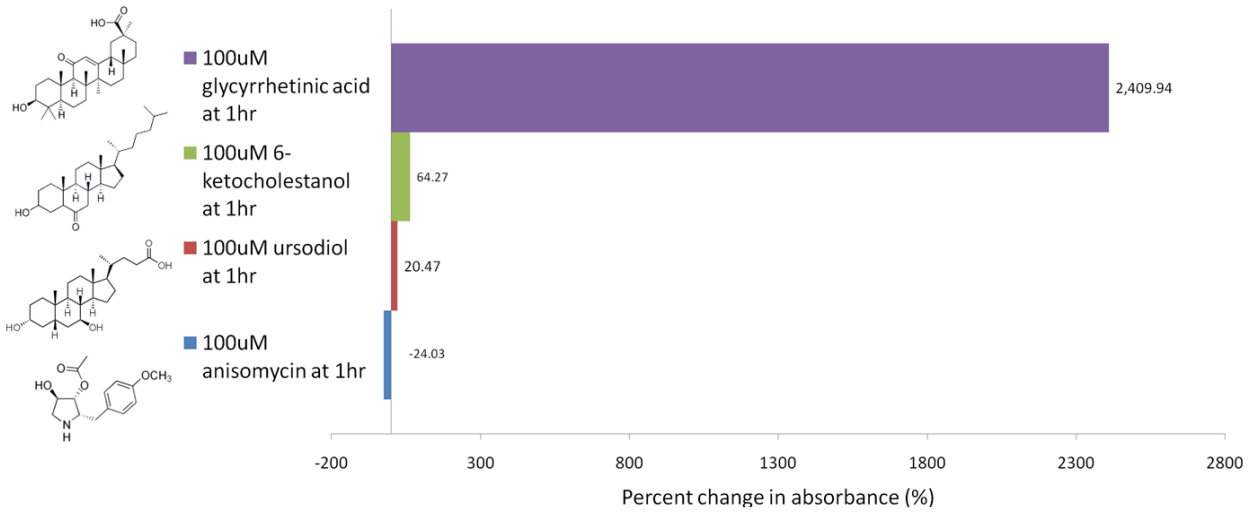


FIGURE 10. Percent change in absorbance with the presence of glycyrrhetinic acid, 6-ketocholestanol and ursodiol.

No.	Sterols	
1	17-a-hydroxy-progesterone	
2	5-cholesten-3b-ol	
3	5a-androstan-3b,17b-diol	
4	17a-hydroxy-pregnenolone	
5	5-a-androstan-3,17-dione	
6	epiandrosterone	
7	dehydroisoandrosterone	
8	D4-androsten-3,17-dione	
9	b-estradiol	
10	testosterone	
11	corticosterone	
12	progesterone	
13	cortisone	
14	estriol	
15	4-cholesten-3-one	
16	cholesterol	
17	hydrocortisone	
18	etiocholan-17b-ol-3-one	
19	estrone	
20	5a-androstan-3a,17b-diol	
21	D5-pregnen-3b-ol-20-one	
22	testosterone acetate	
23	deoxycorticosterone	
24	androsterone	
25	corticosterone-21-acetate	
26	digitonin	
27	5a-cholestan-3b-ol-6-one	
28	5-cholesten-3b,25-diol	
29	5-cholesten-3b-ol-7-one	

Key:

	<10% change in absorbance
	<50% increase in absorbance
	>50% increase in absorbance

FIGURE 11. Screen for Hsp27 oligomerization-inducing sterols. The absorbance was recorded at 300nm after 25μM monomeric Hsp27 samples were incubated with 29 sterols at 100μM. Based on the analysis of percent change in absorbance in 1 hour, sterols can be catagorized into compounds that causes minimal change (<10%) change in absorbance (grey box), that increases absorbance for less than 50% (red box) and that increases absorbance for more than 50% (green box).

Sterols	% increase in absorbance
5a-cholestan-3b-ol-6-one	223.34
4-cholesten-3-one	167.00
estrone	126.50
5a-androstan-3b,17b-diol	92.30
testosterone acetate	90.00
5-a-androstan-3,17-dione	87.80

FIGURE 12. Sterols that significantly induces Hsp27 self-assembly.

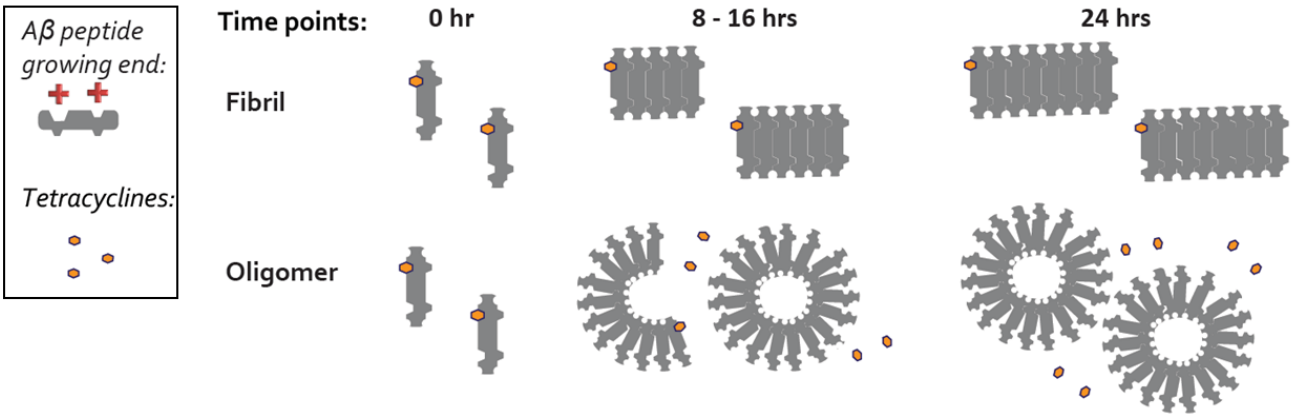


FIGURE 13. Tetracyclines inhibit Aβ peptide self-assembly by binding to the growing end of Aβ The tetracycline binding site at the growing end is available at all time points in elongated fibrils, but the binding site may be hidden once mature oligomer is formed.

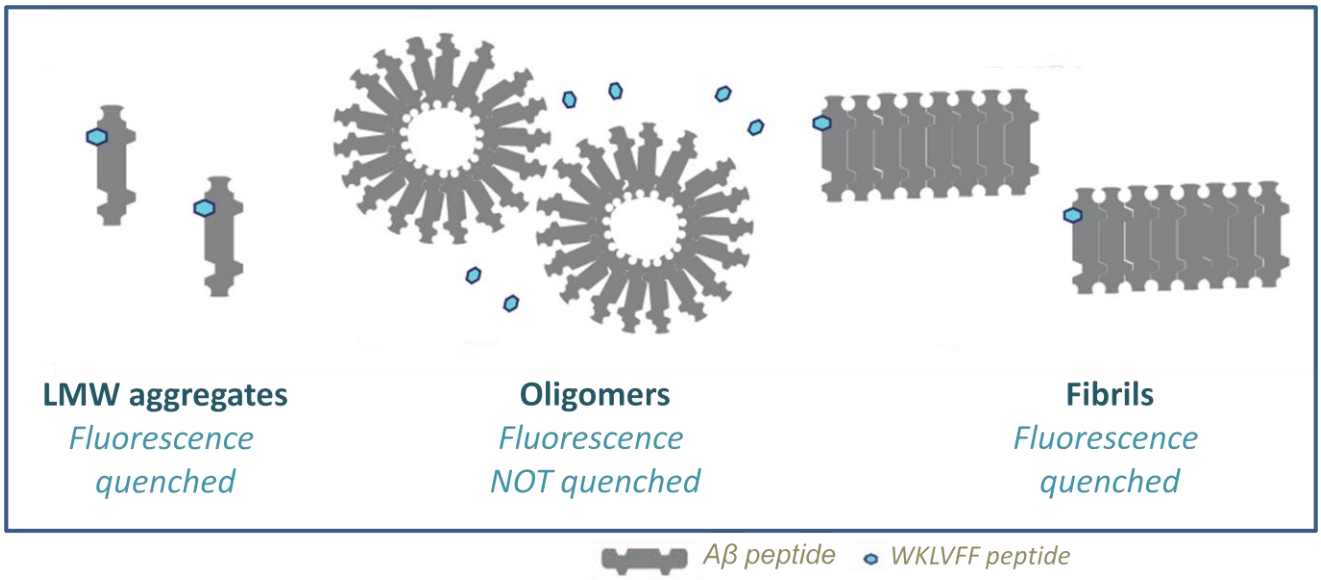
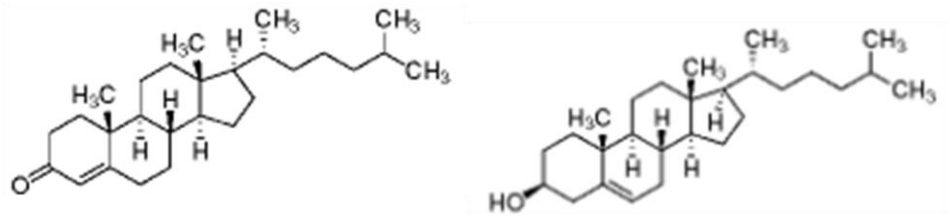


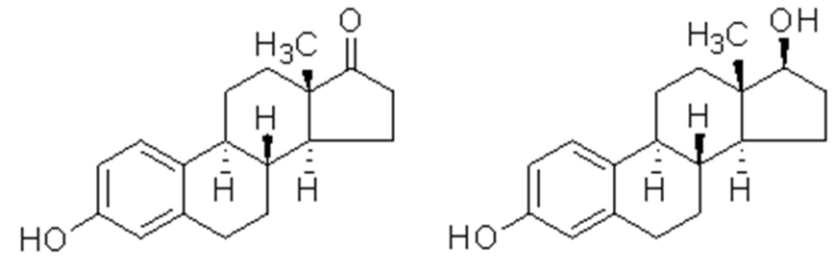
FIGURE 14. WKLVFF binds to KLVFF residues as a growing end binder similar to tetracycline binding pattern. Fluorescence of WKLVFF only quenched when it interacted with LMW aggregates and fibrils, but not with oligomers. This may suggests that KLVFF residues may be partly or entirely buried in pre-formed oligomer



4-cholesten-3-one
% Δ abs = 167%

5-cholesten-3b-ol
% Δ abs = 50%

(A)

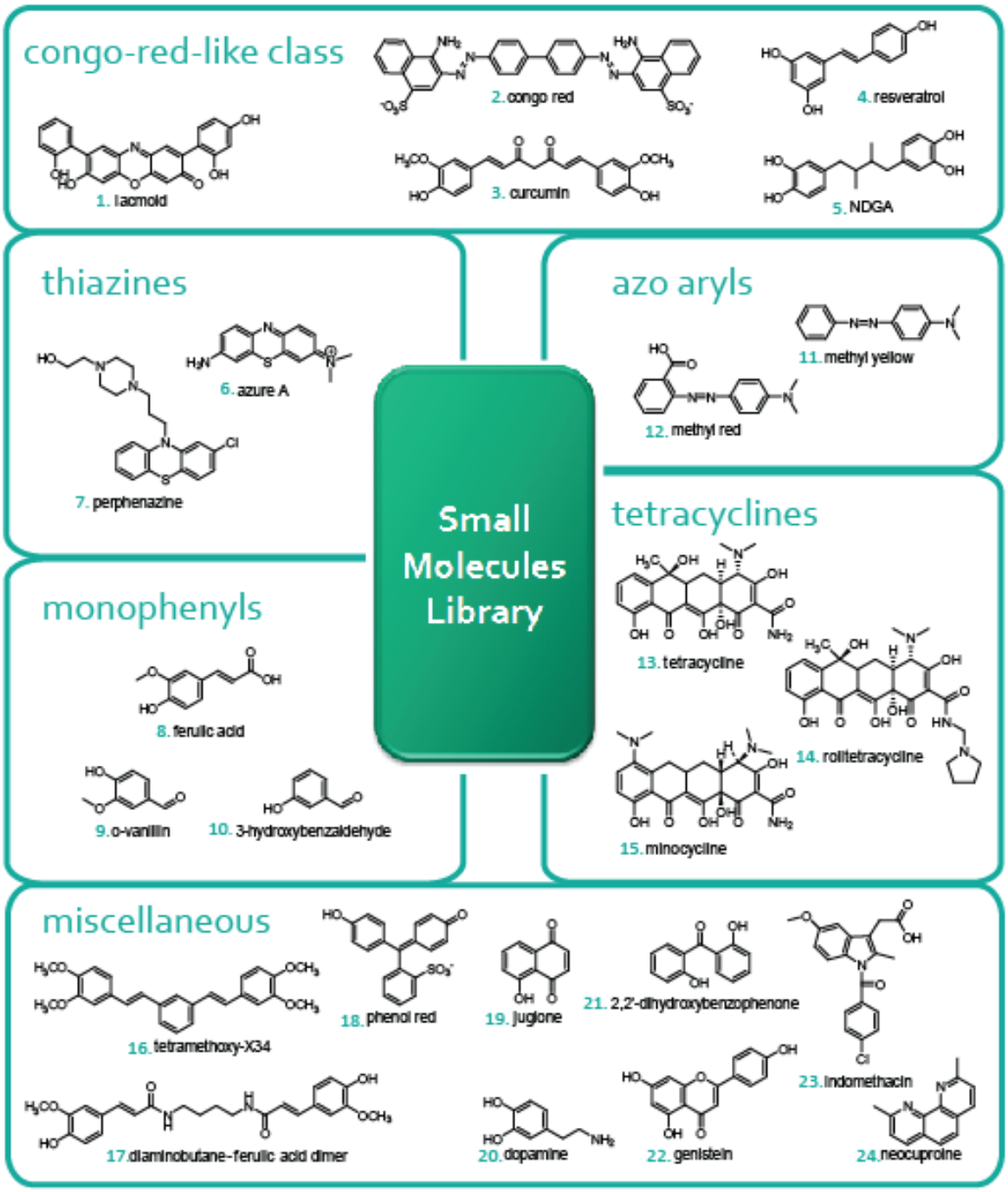


Estrone
% Δ abs = 126.5%

b-estradiol
% Δ abs = -10%

(B)

FIGURE 15. Difference in hydrophobicity of sterols functional group may reduce the ability of sterol in inducing Hsp27 oligomerization. By comparing these chemical structures of sterols replacement of one ketone group with hydroxyl group will greatly. Since ketone is more hydrophobic than hydroxyl group, the result may suggest that the reduction of hydrophobicity of sterol will reduce its ability to bind and interact with Hsp27.



SUPPLEMENTAL FIGURE 1. Small molecules used in Thioflavin T fluorescence assay and their chemical structures.

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