Affinity-Based Inhibition of β-Amyloid Toxicity†

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ABSTRACT: Strategies for interfering with protein aggregation are important for elucidating and controlling the pathologies of amyloid diseases. We have previously identified compounds that block the cellular toxicity of the β-amyloid peptide, but the relationship between their ability to inhibit toxicity and their affinity for Aβ is unknown. To elucidate this relationship, we have developed an assay capable of measuring the affinities of small molecules for β-amyloid peptide. Our approach employs immobilized β-amyloid peptide at low density to minimize the problems that arise from variability in the β-amyloid aggregation state. We found that low-molecular weight (MW of 700−1700) ligands for β-amyloid can be identified readily by using surface plasmon resonance. The best of these bound effectively ($K_d \sim 40$ μM) to β-amyloid. The affinities measured for peptides in the SPR assay correspond to results from Aβ cell toxicity assays. The most potent ligands for immobilized β-amyloid are the most potent inhibitors of the neuronal cell toxicity of β-amyloid. Compounds with dissocation constants above ~100 μM did not show significant activity in the cell toxicity assays. Our data support the hypothesis that ligands exhibiting greater affinity for the β-amyloid peptide are effective at altering its aggregation and inhibiting cell toxicity.

Protein aggregation and amyloid plaque formation are implicated in the pathology of a number of disease states such as Huntington's disease, familial amyloid polyneuropathy (FAP),[1] and Alzheimer's disease (AD) (*1*, *2*). The underlying processes that lead to aggregation in these diseases are poorly understood. In some cases, it is a matter of some controversy whether the formation of amyloid plaques plays a causative role or if it is merely symptomatic (*3*, *4*). Regardless, the development of general strategies for interfering with protein aggregation could have enormous benefits for the development of therapies and the elucidation of the etiology of these diseases.

Alzheimer's disease is a devastating neurodegenerative disorder currently affecting an estimated 4 million people in the United States (*5*). Amyloid plaques found in AD patients contain a 39−42-residue peptide, β-amyloid (Aβ), that is highly prone to aggregation under appropriate conditions (*6*). The natural function of Aβ is unknown. The peptide is found in both the AD and the non-AD brain; however, in the disease state, amyloid plaques containing Aβ are more abundant and are associated with neurodegeneration. A large body of evidence has suggested that the aggregation of Aβ to soluble oligomers or fibrils is important for the development of its toxic effects (*7*−*11*). The findings that mutations associated with familial Alzheimer's disease influence the in vivo concentrations of Aβ or its propensity to form amyloid fibrils provide strong support for the significance of Aβ aggregation (*4*).

Aβ aggregation is an excellent model system for the development of protein antiaggregation strategies for several reasons. (1) The aggregating species, Aβ, is readily available. (2) Both in vitro and in vivo model systems for toxicity of the aggregates have been developed (*12*−*16*). (3) Considerable structural data have been collected on this system (*17*), and compounds that alter aggregation can be used to investigate its role in the disease. These features render Aβ an excellent test case for evaluating general strategies for altering protein aggregation.

Progress in developing therapies for AD has been slow. Emerging approaches are focused on inhibiting the production of Aβ in the brain (*18*, *19*) or removing existing plaques (*20*, *21*). An alternative strategy is to identify small molecules capable of binding Aβ. These compounds could act by disrupting the formation of aggregates and altering aggregate structure, or by inhibiting interactions of Aβ with other receptors (*8*, *22*−*25*).

We hypothesized that compounds with high affinity for Aβ would also serve as effective inhibitors of cellular toxicity. Although some inhibitors of toxicity have been shown to bind to Aβ (*26*, *27*), a clear relationship between the affinity for Aβ and inhibition of toxicity has not been established for any series of compounds. The first step in

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[1] Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; Aha, aminohexanoic acid; EDA, ethylenediamine; EDC, *N*-ethyl-*N*′-[(dimethylamino)propyl]carbodiimide; FAP, familial amyloid polyneuropathy; FMOC, 9-fluorenylmethoxycarbonyl; HBS, HEPES-buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; RP-HPLC, reverse-phase high-pressure liquid chromatography; RU, response units; SAR, structure−activity relationship; sulfo-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; SPR, surface plasmon resonance.

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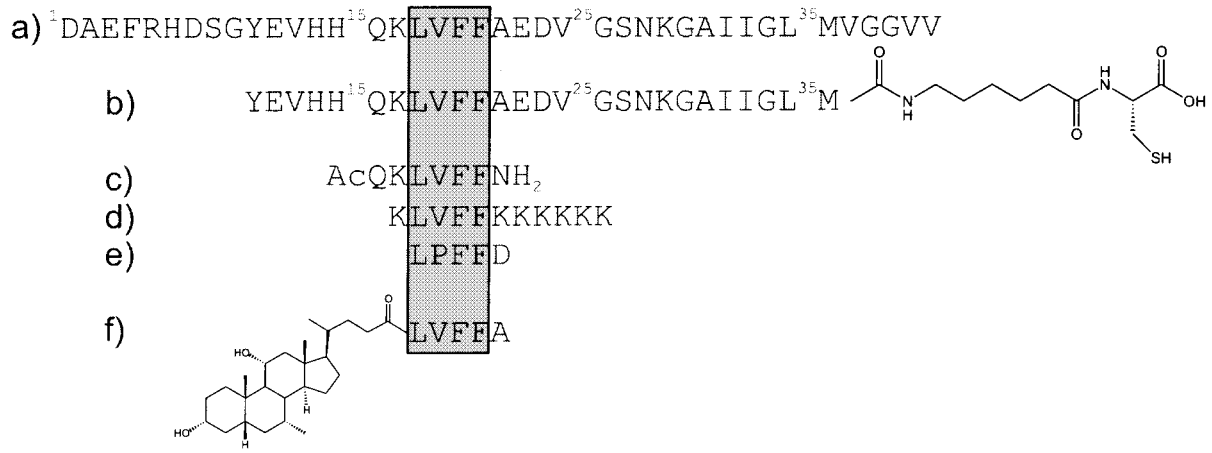

FIGURE 1: Binding target for these studies of the Aβ peptide: (a) the full sequence of Aβ(1−40) and (b) a portion of the native sequence, Aβ(10−35), that was used for SPR binding studies with C-terminal modifications to allow specific immobilization. Homologous binding elements from the Aβ(1−40) sequence have been used by several researchers to design inhibitors of amyloid formation; the Aβ(16−20) region has been used most frequently. Some examples are depicted in parts c−f: (c) Tjernberg et al. (*39*), (d) Ghanta et al. (*40*), (e) Soto et al. (*50*), and (f) Findeis et al. (*44*).

testing our hypothesis required the identification of a method of directly comparing the affinity of a series of compounds for β-amyloid peptide.

The direct binding assays reported to date for screening amyloid inhibitors require extrinsic dyes or the inclusion of either a fluorophore in the inhibitor (*28*) or a radionuclide in Aβ (*29*, *30*). Measurements that employ extrinsic dyes are particularly susceptible to optical artifacts, such as absorbance overlap by ligands and light scattering due to fibril formation. All reported assays consume amyloid peptide for each experiment, and significant effort must be taken to ensure consistent sample preparation (*6*). Approaches that determine the affinity for a single preparation are advantageous and provide an opportunity to readily compare the activities of different compounds. These data could then be used to understand and optimize ligand activity.

We developed a new direct binding assay for identifying small molecules that bind Aβ. Through such an assay, we determined whether compounds with the highest affinities for Aβ are also the most potent inhibitors of amyloid toxicity. Additionally, the mechanism of known inhibitors could be probed and new small molecules that bind Aβ identified. We report the development of a surface plasmon resonance (SPR) assay in which ligand binding to an immobilized form of Aβ can be detected readily.

SPR has been used previously to investigate Aβ interactions; however, these experiments were not designed to identify small molecule binding targets. Tjernberg and coworkers demonstrated a specific interaction of an immobilized peptide with full-length Aβ (*30*). Other studies have focused on observing the growth of immobilized amyloid fibrils (*31*). Neither of these general approaches is amenable to the screening of small molecules. Our strategy was to immobilize an easily handled form of Aβ, Aβ(10−35) (*32*), to a carboxymethyl dextran matrix, and measure the solution affinity of small molecules for this surface. A major advantage of this assay is that the surface density can be manipulated to minimize the density of the binding target, which may also limit its aggregation. Moreover, with SPR detection, no labeling strategy for Aβ is needed. Because a single surface is used for all experiments, there is no target variability between assays. Using this approach, we found that several compounds previously reported to prevent cellular toxicity are also effective ligands for Aβ. Significantly, we demonstrate that the affinity of compounds used in this study corresponds to their ability to prevent the cellular toxicity of Aβ.

EXPERIMENTAL PROCEDURES

Reagents. All reagents were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Peptide Synthesis. Protected peptide residues and resins were purchased from Advanced Chemtech (Louisville, KY). Peptides used in this study were assembled by solid-phase peptide synthesis procedures appropriate for monomers equipped with fluorenylmethoxycarbonyl protecting groups (FMOC). Peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C18 column and a water/acetonitrile mobile phase. All peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectroscopy on a BRUKER REFLEX II mass spectrometer.

Aβ(10−35). Aβ(10−35)-amide used in the initial studies was purchased from QCB Biosource International (Camarillo, CA). The amino acid sequence is YEVHHQKLVFFAEDVGSNKGAIIGLM-NH_2. The reported mass is 2902 Da, and the purity is >97% (purity based on HPLC peak area). The Aβ(10−35)-Aha-Cys (Figure 1b) was synthesized using a Synergy 432A peptide synthesizer (Applied Biosystems, Foster City, CA). The cysteine used for surface immobilization was introduced into the peptide at the C-terminus through an intervening aminohexanoic acid (Aha) spacer. The amino acid sequence is YEVHHQKLVFFAEDVGSNKGAIIGLM-Aha-C. The peptide was purified by RP-HPLC (88% purity based on peak area). The mass of the peptide is 3120 Da, and its sequence was confirmed by amino acid analysis. All protected peptide reagents were purchased from Novabiochem (La Jolla, CA), and the coupling reagents were purchased from Applied Biosystems.

SPR Assay. Sensor chips were purchased from BIAcore (Uppsala, Sweden). All SPR experiments were carried out on a BIAcore 2000 instrument. Reagents for immobilization

(EDC, NHS, and ethanolamine) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The buffer used to immobilize the peptide through amide bond formation was 10 mM sodium acetate (pH 5.0). The regeneration buffer was 4 M guanidine-HCl in 10 mM Tris-HCl (pH 8.0). The running buffer was HEPES-buffered saline (HBS) containing 10 mM HEPES and 150 mM sodium chloride (pH 7.4). All buffers were filtered (0.22 μm, nylon) prior to use. For immobilization via cysteine thiolate addition to maleimide, HBS buffer (pH 7.4) was used for all steps unless otherwise noted. The bifunctional coupling reagent *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) was purchased from Pierce Chemical Co. (Rockford, IL), and ethylenediamine (EDA) and cysteine were purchased from Aldrich and used as provided.

Aβ Immobilization. Attachment of Aβ(10−35)-amide to the CM5 chip followed standard amide bond forming conditions as reported elsewhere (*33*). The flow rate employed for all steps was 5 μL/min to maximize contact time. The carboxymethyl dextran matrix was activated by injection of a 1:1 mixture of *N*-ethyl-*N*′-[(dimethylamino)propyl]-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (70 μL, 200 mM EDC, 50 mM NHS). The β-amyloid peptide-(10−35) fragment was then injected into the activated flow cell (0.5 mg/mL peptide in sodium acetate buffer). Unreacted NHS esters were capped with ethanolamine (70 μL, 1 M, pH 8.5) to afford a surface that gave a final change in response units (RU) of 2700 in the Aβ sample flow cell. The control flow cell was reacted using the same protocol using an injection of ethanolamine buffer (70 μL) instead of the peptide to produce a control cell with reduced charge. The B1 surface was prepared using standard maleimide chemistry (*33*); the surface was activated as described above using NHS/EDC, and then reacted with EDA (70 μL, 1 M, pH 8.5) to generate free amine groups on the surface. Sulfo-MBS (70 μL, 50 mM in HBS) was then injected to generate a surface modified with maleimide groups. For the conjugate addition of the Aβ sequence, the cysteine-containing peptide was dissolved in immobilization buffer containing 10% DMSO at a concentration of 5 mg/mL. The resulting mixture was injected immediately after dilution to afford a final concentration of 50 μg/mL in immobilization buffer with 0.1% DMSO. Cysteine (70 μL, 100 mM in 10 mM NaOAc, pH 5.0) was injected to eliminate free, unreacted maleimide groups. The change in RU corresponding to Aβ immobilization was 1350. The surface was washed with regeneration buffer in short pulses to remove noncovalently associated peptide (20 × 5 μL), and this gave a final response of 800 RU. The control lane was prepared as described above and then blocked with an injection of cysteine after injection of sulfo-MBS.

SPR of Peptide Analytes. Peptide samples were prepared by dilution into running buffer after lyophilization. Each peptide was diluted at 10 concentrations (3000, 2000, 1000, 700, 400, 300, 200, 100, 70, and 50 μM) and injected in multichannel mode (40 μL KINJECT, 5 μL/min). The surface was then exposed to running buffer for 300 s to observe dissociation. The chip surface was regenerated by injection of the regeneration buffer (10 μL). The control lane data were subtracted from raw data obtained from the flow cell with immobilized Aβ. The response at equilibrium (R_{eq}) was then plotted versus concentration (M) using the graphing program Sigma Plot. For the compounds listed in Table 1, the isotherms did not reach a final plateau; thus, the data were fit to a standard binding curve with a fixed R_{max} value (eq 1) (*34*−*36*)

$$R = \frac{R_{max}F}{K_d + F} \quad (1)$$

where R is the response in RU, F is the concentration of free ligand, and K_d is the fitted dissociation constant.

The R_{max} was calculated as the theoretical plateau determined from eq 2, using the molar mass of each analyte (MW_A), the molar mass of the immobilized ligand (MW_L), and the RU of immobilized ligand (RU_L).

$$R_{max} = \frac{MW_A}{MW_L}RU_L \quad (2)$$

This analysis is intended only to provide a relative assessment of binding; it does not yield absolute affinities. This fitting procedure afforded K_{rel} values, a nomenclature used to distinguish them from true affinities. To determine affinities for compounds listed in Table 2, a nonspecific binding term was added to eq 1 (*35*), and data were fit by linear regression to eq 3:

$$R = \frac{R_{max}F}{K_d + F} + K_{ns}F \quad (3)$$

For these analyses, R_{max}, K_d, and K_{ns} were left as independent variables. Alternatively, a model including an additional saturable binding site could be used to fit the data, as in eq 4:

$$R = \frac{R_{max1}F}{K_{d1} + F} + \frac{R_{max2}F}{K_{d2} + F} \quad (4)$$

Cellular Toxicity. All cell culture medium, antibiotics, and serum were purchased from Life Technologies (Gaithersburg, MD). All other chemicals were purchased from Sigma-Aldrich unless stated otherwise. Aβ(1−40) was purchased from AnaSpec, Inc. (San Jose, CA), and used without further

Table 1: SPR Results from Steady State Affinity Determinations of Pentapeptide Ligands

compound	sequence	K_{rel} (mM)[a]	±SE
1	KLVFF	1.4	0.9
2	KLVF	12	8
3	klvff	1.0	0.6
4	KLVFY	1.6	0.6
5	KLVYF	2.4	1.6
6	KLVYY	6	2
7	KLVFH	4	3
8	KLVHF	ND[b]	
9	KLVHH	4	2
10	KLVFW	7	3
11	KLVWF	ND[c]	
12	KLVWW	ND[c]	

[a] Values reported were determined by fitting equilibrium values to a single-site model and assuming the theoretical R_{max} as described in Materials and Methods. The error is reported as the standard error of the fit. [b] K_{rel} could not be determined due to insufficient response levels. [c] K_{rel} could not be determined due to equilibrium values exceeding the theoretical R_{max}.

Table 2: SPR Results from Steady State Affinity Determinations

compound	sequence	K_d (μM)[a]	±SE	% viability[f]
13	KLVFFKKKKKK	40	9	88[c]
14	KLVFFKKKK	37	5	78[c]
15	KLVFFKK	80	40	72[c]
16	KKKKLVFF	180	80	60
17	KLVFFKKKEEE	90	10	69
18	KLVFFEEEKKK	1300	600	62
19	KLVFFEKEKEK	300	160	66
20	KLVFFKEKEKE	ND[b]		62
21	KLVFFRRRRRR	40	10	92
22	KKKKKK	400	200	64
23	KLVWWKKKKKK	40	10	85
24	KLVFWKKKKKK	65	10	74
25	Congo red	38	8	NA[d]
26	VFFAEDVG	ND[b]		NA[e]

[a] Affinity was determined using eq 3, as described in Materials and Methods. The error is reported as the standard error of the fit. K_{ns} values were typically near 70 μM. [b] K_d could not be determined due to insufficient response levels. [c] From ref *47*. [d] From ref *57*. Viability was recovered using a 20:1 molar ratio of Congo red to Aβ(25−35). [e] From ref *40*. Viability was unchanged from that of control Aβ(1−39) in PC12 cells. [f] Untreated Aβ samples gave 59% viability. The standard deviation in all cell viability measurements is ±2%. Viability experiments were performed as described in Materials and Methods unless otherwise noted.

purification. The identity and purity of the peptide were assessed by mass spectrometry and amino acid analysis. The amino acid sequence of the peptide is DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV. The reported molecular weight was 4331.3, and the reported purity was greater than 95.8%. Lyophilized Aβ was stored at −70 °C until it was used. For toxicity experiments, lyophilized Aβ was dissolved in prefiltered 0.1% TFA at 10 mg/mL and then incubated for 1 h at 37 °C. An Aβ stock solution was then diluted to 0.5 mg/mL (115 μM) with sterile-filtered phosphate-buffered saline (PBS) containing penicillin and streptomycin [PBS consists of 0.01 M K_2HPO_4/KH_2PO_4 and 0.14 M NaCl (pH 7.4)]. The samples were allowed to aggregate at 37 °C for 48 h, and then diluted to 25 μM with fresh media for plating. Human neuroblastoma (SH-SY5Y) cells obtained from ATCC (Rockville, MD) were used as model neurons. Cells were stored in liquid nitrogen and thawed in a 37 °C water bath. Cells were cultured to confluence on polylysine-coated T-flasks in medium containing 44% minimal essential medium (MEM) modified to contain 1.5 g/L sodium bicarbonate, 44% Ham's modification of F-12 medium, 10% fetal bovine serum (FBS), 1% L-glutamine (3.6 mM), and 1% penicillin/streptomycin antibiotics (10 000 units/mL). Flasks were incubated in a humidified 37 °C incubator with 5% CO_2. Cells were harvested using 0.4 mM EDTA and 0.05% trypsin, centrifuged at 750*g* for 10 min, and then resuspended in fresh medium, with mild aspiration used to break up clumps. Cells were counted using a hemocytometer (Hausser Scientific) and plated in 96-well polylysine-coated plates with approximately 10 000 cells per 100 μL of medium per well. Plates were incubated in a humidified 37 °C incubator with 5% CO_2 for 24 h to allow cell attachment, and then 80 μL of medium was removed and 80 μL of Aβ or control medium added to cells. Plates were then incubated for an additional 24 h at 37 °C. Cell viability was assessed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] toxicity assay with 20 μL of 2.5 mg/mL MTT in medium per well (*14*). After MTT addition, plates were incubated at 37 °C for 4 h, and then formazan crystals, produced by healthy mitochondria, were dissolved in 100 μL of 50% DMF and 20% SDS (pH 4.7) at 37 °C for 8−12 h. Absorbance at 570 nm was detected with a microplate reader (Bio-Tek Instruments, Winooski, VT) using background subtraction. Cell viability percentages (%*V*) were calculated as follows:

$$\%V = \frac{S - B}{C - B} \quad (5)$$

where *S* is the sample absorbance, *C* is the buffer control absorbance, and *B* is the background absorbance. Sample, background, and control absorbances were averaged over seven replicates. Background samples contained cell culture medium, unreduced MTT, and the SDS/DMF solution.

RESULTS

An assay using an SPR biosensor was developed to evaluate the affinity of small molecule ligands for Aβ. Our initial studies sought to determine whether a specific interaction between Aβ immobilized at low density and small molecules in the desired molecular weight range could be observed. To evaluate whether the necessary sensitivity could be achieved, we immobilized Aβ(10−35) on a carboxymethyl dextran surface (CM5 chip, Biacore AB). We chose Aβ(10−35) as the immobilized target due to its competence to bind to Aβ(1−40) plaques and its consistent aggregation properties (*37*). Under appropriate conditions, Aβ(10−35) has been shown to form aggregates with morphology similar to that of Aβ(1−40) (*32*), and NMR studies suggest that structured aggregates of Aβ(10−35) and Aβ(1−40) are similar (*32*, *38*).

To generate the modified surface required for SPR, the target peptide was linked to the matrix using standard protocols for amide bond formation (*33*). Although this method does not require any modification of Aβ, several lysine residues within the peptide could react; consequently, it is likely immobilized in multiple orientations. Ethanolamine was coupled to a separate activated surface to produce a control lane. The modified surfaces were exposed to solutions containing either of two potential peptide ligands, the pentapeptide KLVFF, Aβ(16−20), and the peptide VFFAEDVG, Aβ(18−25) (Figure 2). These peptides correspond to overlapping regions of the central hydrophobic domain of Aβ, which contains key residues for Aβ−Aβ self-association (*39*). Previously, we reported that Aβ(16−20), but not Aβ(18−25), inhibits the cellular toxicity of Aβ (*40*, *41*). Our SPR experiments revealed that Aβ(16−20) interacted specifically with the immobilized target, but Aβ(18−25) bound poorly. Full-length Aβ(1−40) peptide also bound specifically to immobilized Aβ(10−35) (data not shown). These initial results suggested that specific interactions of ligands with Aβ can be detected using SPR.

Despite the success with our initial binding experiments, we were concerned that Coulombic interactions between charged peptides and the anionic CM5 surface could complicate the evaluation of some ligands. Therefore, to expand the utility of the assay, we selected a surface with different charge density characteristics. Additionally, we

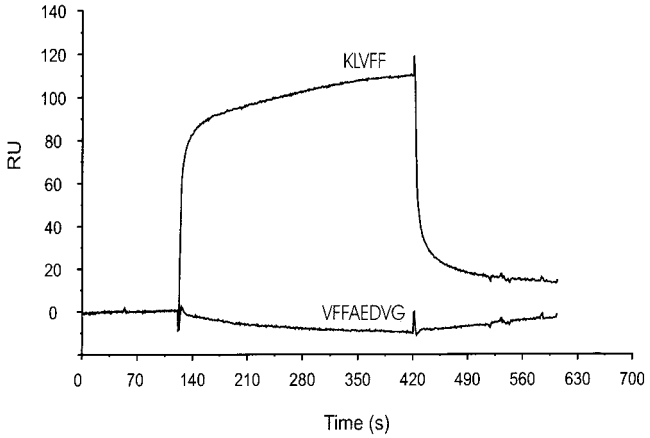

FIGURE 2: Sensorgrams from a flow cell containing a CM5 surface derivatized using amine coupling. Specific interactions were seen with the peptide KLVFF (**1**). VFFAEDVG (**26**) did not exhibit a significant response (responses for all peptides at a concentration of 1 mM in HBS).

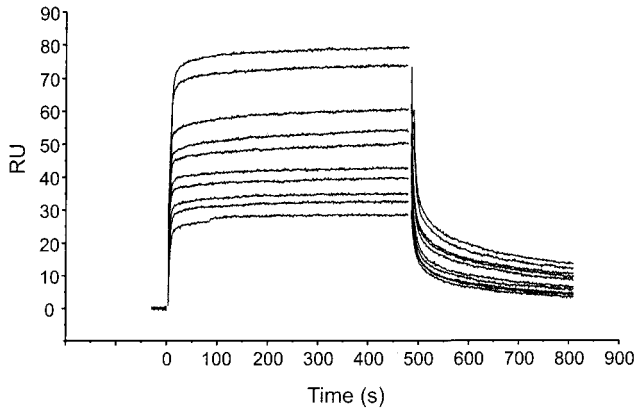

FIGURE 3: Representative set of binding data for $KLVFFK_6$ (**13**). All 10 runs are overlaid for concentrations at 3000, 2000, 1000, 700, 400, 300, 200, 100, 70, and 50 μM. The equilibrium response is determined as the average RU at 90% of contact time (430−440 s) at each concentration. All sensorgrams have control lane data subtracted (subtraction artifacts at the start and end of each sensorgram have been removed for clarity).

employed a selective immobilization chemistry to maximize the uniformity of the binding sites on the surface.

The B1 surface (Biacore AB) is composed of a carboxymethyl dextran matrix with approximately 10-fold fewer carboxylate sites than the CM5 surface. To ensure orientation-specific immobilization of the target, we introduced a C-terminal cysteine residue linked via an aminohexanoic acid (Aha) residue to the Aβ(10−35) sequence (Figure 1b). The cysteine side chain enables selective covalent bond formation to the matrix through conjugate addition of a cysteine thiolate to a maleimide (*33*). To minimize further the likelihood of aggregation on the surface, we immobilized the target at low density. Thus, our conditions for surface modification are designed to minimize the immobilization of aggregates by using (1) a truncated Aβ sequence, (2) orientation-specific immobilization chemistry, and (3) target attachment at low density. The resulting surface was treated with a high-salt denaturant, 4 M guanidine-HCl at pH 8.0, before and after injections of potential ligands to promote conformational homogeneity and dissociate any bound ligands. When Aβ-(16−20) was exposed to this surface, specific binding was observed. Multiple injections of identical samples afforded highly reproducible responses. To test the reproducibility of this procedure, the immobilization protocol was repeated on a fresh sensor chip. This preparation gave similar ligand binding responses and affinity (data not shown). Given the reproducible results obtained with the modified B1 surfaces, we explored the binding interactions of candidate ligands for Aβ.

Several different classes of potential ligands were tested, including peptides and small molecules. First, we examined variants of Aβ(16−20), which contained different aromatic side chains (Table 1). In these variants, one or both of the phenylalanine residues are substituted with tyrosine, tryptophan, or histidine residues. Residues 19 and 20 have been shown previously to be important for plaque formation (*42*), and we sought to determine if these interactions contribute to binding. Second, we tested peptides related to the $KLVFFK_6$ sequence, **13**, in which the C-terminal sequence was varied (Table 2). We demonstrated previously that **13** was a more potent inhibitor of Aβ toxicity than was KLVFF (**1**) (*41*). Thus, compounds designed to explore the importance of the lysine side chains in binding were assayed. Several small molecules previously reported to have effects on the aggregation or biological activities of Aβ were also tested, including Congo red, rifampicin, melatonin, and the pentapeptide LPFFD (*23*).

We analyzed the affinities of our ligands using reference-subtracted response levels at equilibrium to determine the binding isotherm (Figure 3) (*34*). In the case of low-affinity ligands, we were able to obtain approximate fits using a single-site model (eq 1) by assuming the curves would reach a similar plateau. The relative affinity of these compounds could be compared using this method due to their similar mass and structure. This method is similar to that employed in other studies that determined the relative affinities of related compounds (*36*). It provides only a relative assessment of binding and should not be directly compared to dissociation constants. The results from this analysis (Table 1) were similar to those obtained using graphical extrapolation methods for determining relative affinities (*43*).

A series of variants of the KLVFF sequence were tested in the SPR assay. Truncation of the C-terminal phenylalanine (**2**) reduces affinity by approximately 10-fold. The D-amino acid sequence klvff (**3**) bound with similar affinity to **1**, as might be expected from previous reports (*30*, *44*). Substitutions of tyrosine at either the 19 or 20 position (**4** and **5**) did not alter the affinity; however, replacement of both phenylalanines with tyrosine (**6**) was detrimental. Substitution of histidine in position 19 (**8**), but not in position 20 (**7**), led to a substantial loss of binding; nevertheless, a double histidine substitution (**9**) partially restored binding. Substitution of tryptophan for phenylalanine residues gave mixed results. The sequence with a tryptophan residue at position 20 was less potent (**10**). When the analogous change was made at position 19 (**11**) or when substitutions were made at both positions (**12**), the resulting peptides afforded R_{eq} levels well above the theoretical R_{max} at high concentrations. This finding suggests that these peptides aggregate in solution at high concentrations. Thus, the data from these compounds cannot be analyzed using a theoretical R_{max}. Still, the results demonstrate that compounds that interact by different mechanisms may be identified by this method. For compounds

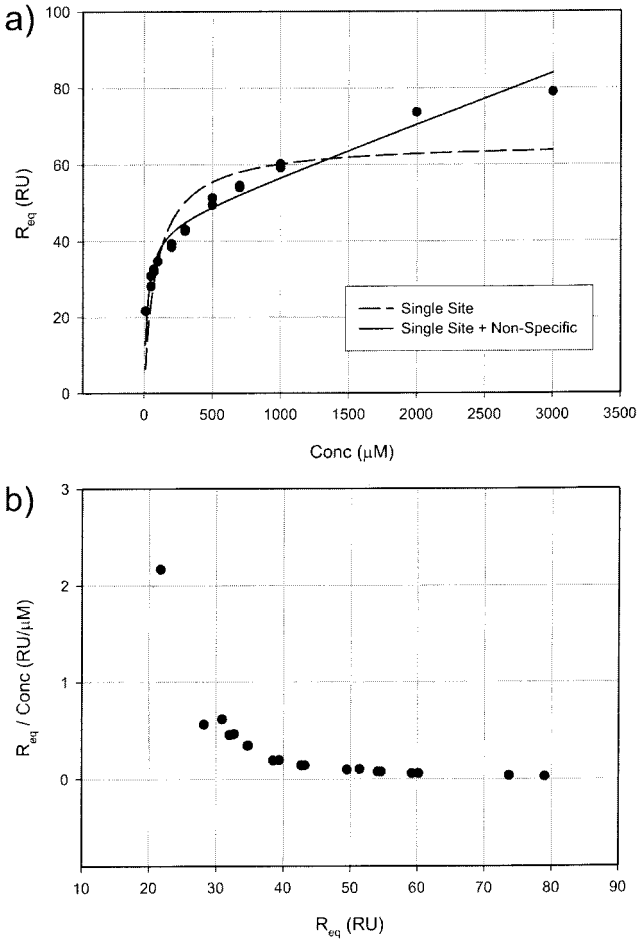

FIGURE 4: Equilibrium analysis of binding data for $KLVFFK_6$ (**13**). (a) Regression analysis of binding affinity using the equilibrium values. Both single-site (eq 1) and single-site with nonspecific term (eq 3) models are shown. (b) A linear transform of the equilibrium data for a Scatchard plot indicates heterogeneity in binding.

with higher binding activities, an analysis could be employed to determine absolute affinities.

KLVFF sequences that also possess positively charged residues at the C-terminus almost invariably bind with higher affinities to immobilized Aβ(10−35) than does KLVFF alone. The binding isotherms for the former class of compounds suggested that a single-site model was not appropriate for analysis of the data from these ligands (Figure 4). The Scatchard plot (*45*) indicated a clear nonlinear dependence. Given our selective immobilization strategy, it is unlikely that multiple orientations of the Aβ target are responsible for the observed heterogeneity. It likely arises from an additional weak binding site contained within the immobilized sequence. This multiplicity of binding sites has been detected previously in studies that examined the binding of radiolabeled Aβ(1−40) to short homologous peptides (*39*). In addition to the identification of the primary Aβ self-recognition sequence as Aβ(16−20), a secondary site was also found within the Aβ(24−34) sequence. We suspect that this region provides an additional weak binding site within the immobilized peptide that accounts for the observed heterogeneity. We therefore analyzed binding isotherms for compounds **13**−**25** using two models that could account for the presence of an additional site. The simplest model includes a single term to account for a nonspecific binding site. Treatment of the data using the single-site model with a nonspecific term (eq 3) instead of the single-site model (eq 1) showed a large improvement in the data fits (the sum of the squares of the residuals was reduced by as much as 4−30-fold). An alternative model that could also account for the observed heterogeneity uses an independent second binding site (eq 4). Treatment of the data using this independent two-site model (eq 4) in place of the single-site model with a nonspecific site (eq 3) did provide an improved fit for some compounds, but the relative improvement was diminished from that given by eq 3. Therefore, the samples were analyzed using the single-site model with a nonspecific term (eq 3).

The K_d values determined from our analysis are summarized in Table 2. Compound **13** ($KLVFFK_6$) is an effective ligand for Aβ (K_d = 40 μM). This result indicates that a peptide domain lacking direct homology with Aβ can play a significant role in binding. The increased affinities of sequences bearing lysine residues are not due to nonspecific Coulombic interactions of $KLVFFK_6$ with the surface, as the affinities of compounds **17**−**20** and **22** indicate. Comparison of the affinities of compounds **13**−**15** reveals that increasing the number of lysine residues from four to six does not afford more potent ligands. The position of the positively charged residues has a critical influence on Aβ affinity. Placement of the positively charged residues close to position 20, as in **17**, gives rise to the most potent ligand of the four isomers. Placing the lysines three residues apart from the region of residues 16−20 with intervening nega-

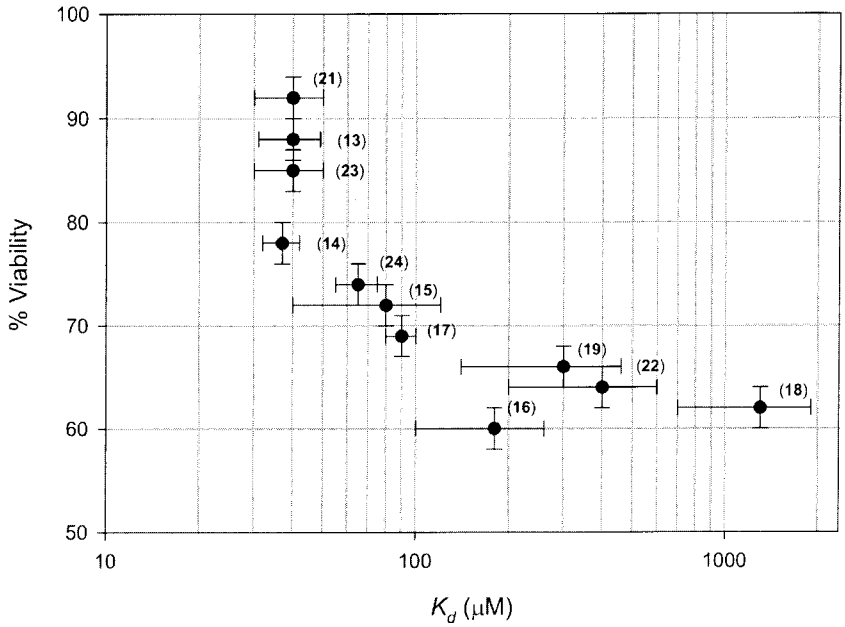

FIGURE 5: Correlation of affinity measured by SPR with prevention of cellular toxicity of Aβ(1−40). For toxicity experiments, the final Aβ concentration was 25 μM, and the Aβ:peptide molar ratio was 1:1. Cellular viability was assessed with the MTT assay (*14*). Each value represents the mean of results from two separate runs with seven replicates per run.

tively charged residues (**18**) reduces the affinity by as much as 14-fold. Placement of positive charge at the N-terminus of the sequence of residues 16−20, as in **16,** results in an activity that is lower than that of compounds **13**−**15**.

Arginine-containing compound **21** was tested to determine the potency of compounds that incorporate positively charged residues other than lysine. These compounds possess activity similar to that of the lysine-displaying compound **13**, suggesting that appending other positively charged sequences can afford compounds that exhibit enhancements in affinity relative to KLVFF. To examine the effects of altering the Aβ(16−20) region in the context of a composite sequence, compounds **23** and **24** were tested. The activity of compound **23** is similar to that of **13**; however, **24** is less potent than would be expected from the relative activity of **10**.

Measurements of the affinities of several small molecules previously reported to alter fibrillogenesis and in vitro toxicity were also conducted. We observed insignificant response levels (<10 RU) for melatonin, rifampicin, and the peptide sequence LPFFD. Congo red, however, did bind effectively to immobilized Aβ in this assay. It was found to have reasonable affinity, although lower than that reported for aggregated Aβ (Table 2).

To examine the ability of the more potent Aβ ligands to influence cellular toxicity, we examined their potency using the MTT assay (*14*). A decrease in the level of MTT reduction, which indicates loss of mitochondrial function, is an early indicator of Aβ-mediated toxicity (*46*). We have previously used this assay to show that compounds related to **13** are effective inhibitors of Aβ toxicity (*40*, *41*, *47*). The results of these experiments are given in Table 2, and they show excellent agreement with the measured affinities from SPR (Figure 5). Compounds with measured dissociation constants lower than approximately 50 μM (**13**, **14**, and **21**) afforded protection against toxicity with cell viability levels of >80%. The compounds (**16**, **18**−**20**, and **22**) that were less effective ligands in our binding assay (i.e., with K_d values of >100 μM) were less effective at preventing the cellular toxicity of Aβ.

DISCUSSION

The development of strategies for altering protein aggregation is important for understanding and treating amyloid diseases. One approach to this problem is to identify compounds that bind the target protein, because these might interfere with its aggregation and toxicity. This strategy is based on the underlying assumption that compounds interact with the target protein to mask sites that would otherwise be accessible for homotypic protein−protein interactions. In the case of transthyretin, compounds that stabilize the folded state have been found to inhibit transthyretin aggregation (*48*). For targets with less defined structures, such as Aβ, a reasonable hypothesis is that compounds that can bind the key regions involved in aggregation might serve as effective modulators of the aggregation process.

We reasoned that compounds that bind Aβ would be likely to alter its aggregation pathways and thereby prevent its toxicity. Compounds with these characteristics serve as useful probes of the molecular mechanisms underlying amyloid formation and pathology and as leads for the design of therapeutic agents. Previously, we and others have identified compounds that alter Aβ−Aβ association processes (*30*, *39*−*41*, *44*, *47*, *49*−*51*). Although some of these compounds have been found to block the cellular toxicity of Aβ, the relationship between Aβ binding affinity and inhibition of toxicity has been obscure (*27*).

To test our hypothesis that the most effective ligands for Aβ would be the most effective inhibitors of its toxicity, we developed an assay for determining relative affinities of a series of compounds for Aβ. Using an immobilized form of a target protein fragment Aβ(10−35), the relative affinities for small molecules can be assessed with SPR. A key to this success is an immobilization protocol that provides a consistent preparation of the target, Aβ(10−35). In any

solution-based assay, Aβ monomers readily aggregate to generate a diverse mixture of different targets. By immobilization of a form of Aβ at low density, a surface that affords consistent binding results is obtained. Another benefit of our approach is that the surface modified with Aβ(10−35) is stable for several weeks and can be used for multiple assays. With this assay method, the relative affinities of several groups of small molecules were measured and compared to their ability to prevent Aβ cellular toxicity.

Among the tetrapeptide and pentapeptide ligands that were screened, no sequences were found with greater potency than the original Aβ(16−20) sequence, KLVFF. It has been observed previously that this ligand is capable of altering Aβ aggregation and toxicity (*39*−*41*). We sought to determine if substitutions of the aromatic side chains might give rise to sequences with altered affinities. Compounds **1**−**12** all contained minor permutations of the Aβ(16−20) sequence (Table 1). Because these variants bound weakly to immobilized Aβ, only their relative binding abilities could be evaluated. Many variations of the aromatic residues resulted in a decreased level of binding relative to **1** (KLVFF). These data indicate that the phenylalanine residues contribute to the ability of the KLVFF sequence to bind Aβ. Sequences with conservative changes at these positions, however, retain activity. Still, we were unable to find more potent sequences with standard amino acid substitutions. Our results suggest that peptidomimetic strategies are required to discover more potent analogues. To examine the relation between affinity and the ability of small molecules to inhibit Aβ toxicity, we explored composite sequences with greater affinity for Aβ.

We reported previously that composite peptides containing short sequences composed of hydrophilic amino acids appended onto the Aβ(16−20) fragment are effective at inhibiting Aβ toxicity (*40*, *41*, *47*). The affinity of these composite sequences (comprising an Aβ recognition element appended to a more hydrophilic sequence) for Aβ is enhanced by positively charged residues at the C-terminus (Table 2). The differences in affinity between the composite sequences containing additional lysine residues, compounds **13**−**18**, reveal that the improvements in activity are due to the specific interactions of the C-terminal lysine residues with Aβ. The placement of the lysine residues within a sequence greatly influences its binding affinity for the target. We postulate that these residues engage in complementary Coulombic interactions with negatively charged residues in the target sequence (e.g., E22 and D23). In accord with this model, the arginine-containing peptide **21** bound to immobilized Aβ with an affinity identical to that of its lysine-substituted counterpart **13**.

The increased affinities of compounds **13**, **14**, and **21** for Aβ relative to KLVFF may be due to the binding of these peptides in a parallel β-sheet mode. Parallel as well as antiparallel binding modes have been invoked for assemblies of Aβ-derived sequences (*2*, *32*, *38*, *52*, *53*), suggesting that the specific sequence that is investigated might determine the binding mode. Additionally, cross-β-sheet interactions involving charge−charge interactions can be exceptionally favorable (*54*). The lysine residues of compounds **13** and **14**, for example, could make favorable contacts with residues E22 and D23 within the target in the parallel mode. The arginine residues within peptide **21** would be expected to interact similarly. Interestingly, such contacts for compound **16** would only be accessible through an antiparallel binding mode, and compound **14** binds more tightly to Aβ than does **16**. Further characterization of the binding modes for different peptides will facilitate the optimization of Aβ ligand structure.

The finding that the highest-affinity compounds indicate a synergistic contribution of the hydrophobic recognition element (LVFF) and a positively charged sequence (oligolysine or -arginine) provides new directions for improving the affinity of Aβ ligands. The results suggest new sites for interaction that can be used to generate compounds that make multipoint contacts with Aβ. In addition, composite sequences that contain recognition elements beyond the KLVFF sequence can bind with high affinity. Compounds **23** and **24** contain changes in this recognition sequence, and these are effective inhibitors of toxicity.

The data for Congo red indicate that the SPR assay can report on the affinities of nonpeptidyl small molecules for Aβ. Additionally, the interactions of Congo red with immobilized Aβ measured here provide indirect evidence for site isolation of the Aβ target on the surface (Table 2). Congo red has been reported to interact with both monomeric Aβ and fibrillar aggregates (*55*). Our experiments with immobilized Aβ result in a substantially weaker affinity for Congo red than that reported for binding to aggregated fibrils (*56*). Thus, the affinity of a ligand can be highly dependent upon the aggregation state of the target. This result highlights the advantages of our surface-based approach, and its potential to determine binding affinities for specific aggregation states.

A key finding from our results is that a direct binding assay can identify compounds that alter Aβ toxicity. Several of the compounds examined here were previously identified as inhibitors of toxicity [i.e., compounds **1**, **13**−**15**, and **25** (*23*, *41*, *47*)]. Our results suggest that their mechanism of action depends on high-affinity binding to Aβ. Not all compounds reported to interfere with Aβ toxicity were found to bind to the immobilized Aβ species in this assay. For example, several other compounds with reported biological activity were ineffective in our assay (i.e., melatonin, rifampicin, and LPFFD). Their lack of activity in this assay suggests that these compounds may act by a different mechanism. Alternatively, these compounds may not bind effectively to the Aβ(10−35) sequence used here, or they may only interact with higher-order assemblies of Aβ, which we have attempted to minimize on our surface. In addition to providing leads for inhibitors of toxicity, our ability to directly monitor binding to Aβ allows us to dissect the role of Aβ affinity in inhibitor function.

Our direct binding assay for determining the affinity of small molecules for Aβ serves as a highly effective method of identifying compounds that bind Aβ. Significantly, the SPR assay is convenient, is reproducible, and has the necessary sensitivity to detect the interaction of low-molecular weight ligands with the Aβ target. Our finding that ligands for immobilized Aβ are effective at preventing Aβ toxicity in cell culture provides support for the hypothesis that ligands for Aβ can function as effective inhibitors of its toxicity. The assay we describe provides the means of identifying such inhibitors.

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