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# Synthesis and evaluation of ferrocenoyl pentapeptide (Fc-KLVFF) as an inhibitor of Alzheimer's $A\beta_{1-42}$ fibril formation in vitro

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ABSTRACT

Aggregation and fibril formation of β-amyloid peptides (Aβ) is the key event in the pathogenesis of Alzheimer's disease. Many efforts have been made on the development of effective inhibitors to prevent Aβ fibril formation or disassemble the preformed Aβ fibrils. Peptide inhibitors with sequences homologous to the hydrophobic segments of Aβ can alter the aggregation pathway of Aβ, together with decrease of the cell toxicity. In this study, the conjugate of ferrocenoyl (Fc) with pentapeptide KLVFF (Fc-KLVFF), was synthesized by HBTU/HOBt protocol in solution. The inhibitory effect of Fc-KLVFF on $A\beta_{1-42}$ fibril formation was evaluated by thioflavin T fluorescence assay, and confirmed by atomic force microscopy (AFM) and transmission electron microscopy (TEM) analyses. Fc-KLVFF shows high inhibitory effect towards the fibril formation of $A\beta_{1-42}$. Additionally, the attachment of ferrocenoyl moiety onto peptides allows us to investigate the interaction between the inhibitor and $A\beta_{1-42}$ in real-time by electrochemical method. As expected, tethering of ferrocenoyl moiety onto pentapeptide shows improved lipophilicity and significant resistance towards proteolytic degradation compared to its parent peptide.



Alzheimer's disease (AD) is the most common cause of dementia and there is so far no clinically accepted treatment to cure it or to halt its progression. In the past two decades, tremendous research efforts have been made to understand the pathogenesis of AD, thus providing a platform to develop effective pharmacological treatments. Although the precise mechanisms of neurodegeneration in AD are not yet fully discovered, several pieces of evidence indicate that the conformational changes along with the aggregation and fibril formation of beta-amyloid (Aβ) might play a pivotal role in the pathogenesis of AD. The Aβ peptide, a 39–43 residue amphipathic peptide, is formed after sequential cleavage of the amyloid precursor protein (APP), by β- and γ-secretase. Understanding the aggregation process and developing agents that can inhibit Aβ fibril formation or disassemble the preformed toxic Aβ fibrils is at the forefront of treatment for those suffering from Alzheimer's disease. A lot of progress has recently been made in the design of inhibitors for Aβ fibril formation and/or misfolding.

To date, lots of structurally different compounds have been identified to prevent Aβ aggregation or disassemble the preformed fibrils. For example, some small organic molecules, such as the dye Congo red,[1] apomorphine derivatives,[2] the antibiotic rifampicin and its derivatives[3] were found to inhibit or reduce the aggregation and toxicity of Aβ in vitro. Especially, in recent years, many modified peptides or peptidomimetics derived from the parent Aβ have been synthesized as inhibitors towards Aβ aggregation.[4] Among the peptide inhibitors, KLVFF, which contains the hydrophobic core of $A\beta_{1-42}$ and can interact with the corresponding residues of Aβ via self-cognition, shows high inhibitory effect for Aβ fibril formation and disassembles the preformed Aβ fibrils.[5] Thus, KLVFF sequence was widely employed as a leading compound for the development of inhibitory agents for Alzheimer's disease.[5,6] However, the inherent properties of KLVFF, such as poor stability against enzymatic degradation and low lipophilicity, hinder its application as an inhibitor for Aβ fibril formation. Lots of approaches were proposed to improve such properties of KLVFF. For example, Findeis et al. added a bulky group to the N-terminus of the peptide motif, and designed the peptide analogues cholyl-LVFFA-OH (PPI-368), which showed significantly improved activity in inhibiting aggregation of Aβ in vitro.[7]

Ferrocene and its derivatives exhibit manifold medicinal properties such as antimalarial,[8–10] antitumor[11,12] and antibacterial properties of ferrocenyl compounds,[13,14] due to their biological activity, low cytotoxicity, high lipophilicity and unique electrochemical behavior.[15,16] In particular, when incorporated into a drug, ferrocene moiety could yield interesting results. For instance, Metzler-Nolte and co-workers synthesized ferrocene-[Leu(5)]-enkephalin conjugate and found that the introduction of the ferrocene moiety enhanced the uptake into cells and the

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permeation coefficient of [Leu(5)]-enkephalin.[17] Jaouen and co-workers attached the ferrocene moiety into an anticancer drug tamoxifen.[18]

In order to combine the lipophilic nature, proteolytic stability and the unique electrochemical behavior of ferrocenoyl moiety and inhibitory characteristic of the pentapeptide KLVFF, we designed a ferrocenoyl attached peptide Fc-Lys(Z)-Leu-Val-Phe-Phe-OMe (Fc-KLVFF) (see Fig. 1). The inhibitory effects of the ferrocenoyl conjugate on $A\beta_{1-42}$ aggregation were investigated by thioflavin T (ThT) fluorescence assay, and confirmed by AFM, TEM analyses and electrochemical method in vitro.

The ferrocenoyl attached pentapeptide Fc-KLVFF, corresponding to the residues 16–20 of Aβ, was synthesized using standard Boc protecting group and activated by HBTU/HOBt strategy in solution following the reported procedures.[19,20] The identity of the ferrocenoyl peptide was confirmed by ESI-MS, NMR, UV–vis and FT-IR. The proton and carbon chemical shifts of NMR for Fc-KLVFF were assigned by analysis of COSY, HSQC, $^{13}C$ and HMBC spectra (for details see Supplementary data).

The binding of ThT dye to amyloid is specific and generates a shift in the emission spectrum of ThT and a fluorescence enhancement proportional to the amount of amyloid.[21] Thus, ThT fluorescence assay was utilized to quantify the inhibitory effect of the compounds on $A\beta_{1-42}$ fibril formation in vitro. All the measurements were carried out under the same experimental conditions as described in Supplementary data. The kinetics of fibril formation of $A\beta_{1-42}$ was examined by ThT fluorescence in the absence or presence of each inhibitor for 12 days at 32 °C. The inhibitory effect of inhibitors on $A\beta_{1-42}$ fibril formation is presented in Figure 2.

As depicted in Figure 2, the freshly solubilized 10 μM $A\beta_{1-42}$, when incubated alone at 32 °C, shows a gradual increase in fluorescence signal. The fluorescence intensity reaches its plateau at the sixth day of incubation. When $A\beta_{1-42}$ is incubated in the presence of 40 μM of KLVFF, the fluorescence intensity increases and reaches its maximum at the fourth day. However, the maximum fluorescence intensity of $A\beta_{1-42}$ in the presence of KLVFF is much lower than that of $A\beta_{1-42}$ alone. After that, a slight decrease of the fluorescence intensity in the presence of KLVFF was observed. Fc-KLVFF exhibits similar ThT fluorescence pattern as that of KLVFF. Both KLVFF and Fc-KLVFF reveals significantly inhibitory effects, resulting in 89% and 93% reduction of the fibril formation, respectively. Our results point to robust inhibitory effects of Fc-KLVFF toward $A\beta_{1-42}$ fibril formation. In addition, we tested the dose–response relationship to the inhibitory effect. As shown in Figure 3, the inhibitory effect enhances rapidly with increasing dosage of inhibitors. The $IC_{50}$ values (the molar ratio of inhibitor to $A\beta_{1-42}$ with 50% maximum effect) for Fc-KLVFF and KLVFF are 0.18 and 0.33, respectively. The results clearly indicate that both Fc-KLVFF and KLVFF have excellent inhibitory effects on $A\beta_{1-42}$ fibril formation.

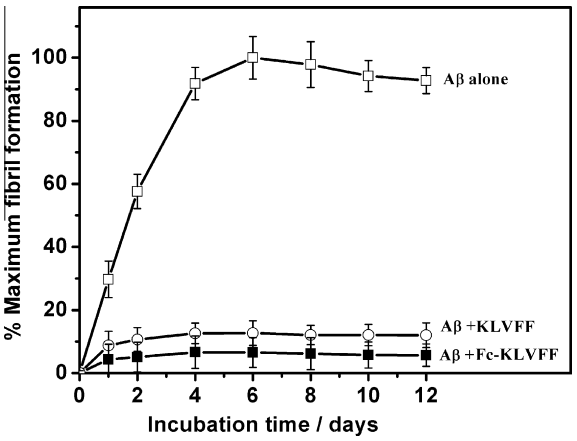


**Figure 2.** Kinetics of KLVFF and Fc-KLVFF inhibition on $A\beta_{1-42}$ fibril formation using ThT fluorescence assay. The freshly solubilized 10 μM $A\beta_{1-42}$ was incubated at 32 °C alone (□), or in the presence of 40 μM KLVFF (O) and 40 μM Fc-KLVFF (■) in a mixed solvent of DMSO and water (3:1, V/V) for the indicated times. Fibril formation was quantified by measuring fluorescence intensity. Data were expressed as percentage of maximum fibril formation (fluorescence intensity). Each experiment was repeated three times ($n = 3$). Error bars represent the standard deviation (SD) of the fluorescence measurement.

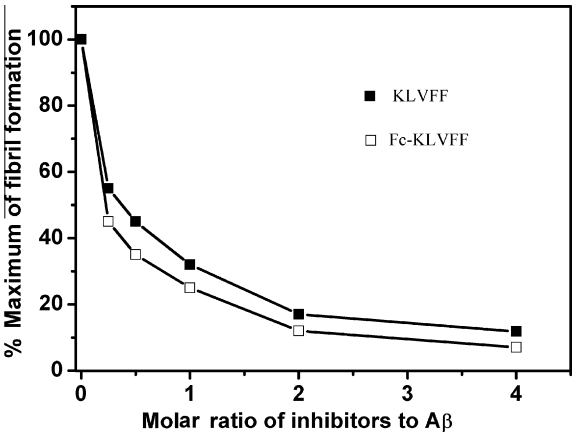


**Figure 3.** Dose–response inhibition of inhibitors on $A\beta_{1-42}$ fibril formation. The freshly solubilized 10 μM $A\beta_{1-42}$ was incubated at 32 °C in the presence of KLVFF(■) and Fc-KLVFF (□) of difference concentrations in a mixed solvent of DMSO and water (3:1, v/v) for four days. Fibril formation was quantified by measuring fluorescence intensity.

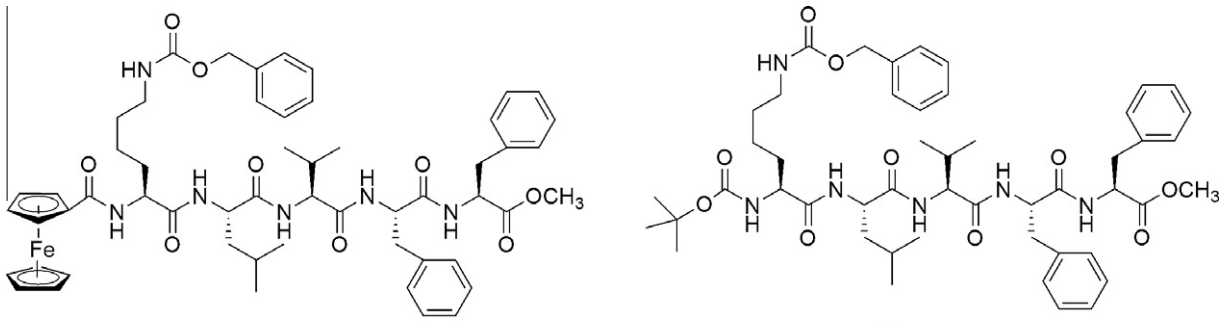


**Figure 1.** Structures of KLVFF and Fc-KLVFF.

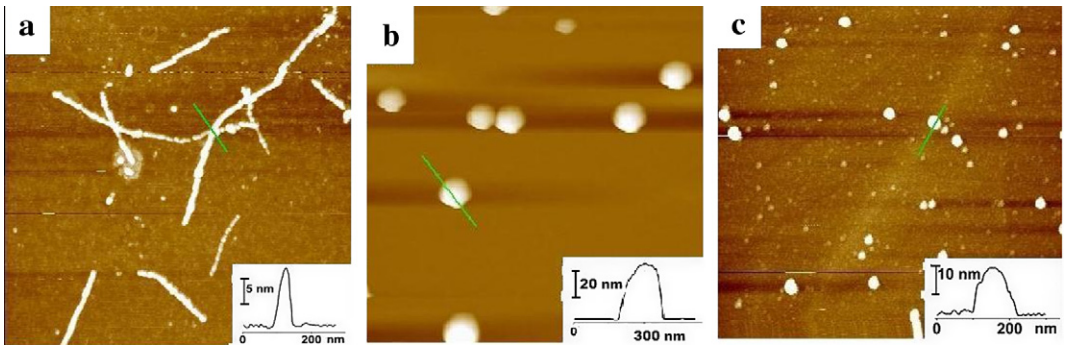


**Figure 4.** AFM images of 10 μM $A\beta_{1-42}$ incubated at 32 °C for 7 days in the absence (a) or in the presence of 40 μM Fc-KLVFF (b), and 40 μM KLVFF (c) in a mixed solvent of DMSO and water (3:1, v/v). The scale of each image is 2 × 2 μm. The cross-sectional contours showed the representative aggregates identified by the line.

Furthermore, a control experiment was performed to test the amyloid-like fibril formation of Fc-KLVFF alone using ThT fluorescence assay. A 40 μM Fc-KLVFF or KLVFF was incubated alone for 7 days at 32 °C, respectively. No obvious ThT fluorescence was observed in either solution (data no shown), which suggests that neither KLVFF nor Fc-KLVFF undergoes amyloid-like fibril formation in solution.

AFM and TEM were employed to confirm the inhibitory effect of Fc-KLVFF on Aβ fibril formation. As revealed by Figure 4a, in the absence of inhibitor, $A\beta_{1-42}$ formed visible amyloid-like, 6–10 nm in diameter (or height) fibrils. In contrast, in the presence of 40 μM Fc-KLVFF, only a few insoluble globular aggregates were observed but no protofibrils or any kind of fibrillar materials were shown (cf. Fig. 4b). We also found that the incubation of $A\beta_{1-42}$ in the presence of 40 μM KLVFF yields amorphous aggregates, but the height of the amorphous deposit is lower than that in the presence of Fc-KLVFF (cf. the cross-sectional contours of representative aggregates were given below the AFM images in Figure 4). The AFM results are in striking agreement with the results reported by Etienne et al. who used modified KLVFF (AMY-1 and AMY-2) as inhibitors.[22] In our work, as shown in Figure 5, Fc-KLVFF and KLVFF are able to inhibit $A\beta_{1-42}$ fibrillization even after 5 months of incubation at room temperature. TEM images of $A\beta_{1-42}$ incubated in the presence of Fc-KLVFF at room temperature for 5 months displayed no signs of fibril formation but rather only globular, non-fibrillar protein aggregates (Fig. 5a). In the same condition, $A\beta_{1-42}$ incubated with KLVFF also displays no identifiable fibrils but only amorphous aggregates (Fig. 5b). The AFM and TEM analytical results are in good agreement with ThT fluorescence.

To obtain further evidence for the interaction of the Fc-KLVFF with the $A\beta_{1-42}$, we utilized the electrochemical method to study the interaction between Aβ and Fc-KLVFF according to the redox-active properties of ferrocenoyl conjugate. The electrochemical behavior of Fc-KLVFF was investigated by cyclic voltammetry (CV). Fc-KLVFF exhibits a reversible electrochemical one-electron redox, with the ratio of oxidative to reductive peak currents of close to unity. The cathodic and anodic peak potentials were observed at 0.546 and 0.624 V versus Ag/AgCl, respectively. In addition, the kinetics of $A\beta_{1-42}$ fibril formation in the presence of Fc-KLVFF was calculated by conducting differential pulse voltammetry (DPV). As shown in Figure 6, the oxidation current decreases sharply in a very short time when Fc-KLVFF is incubated with $A\beta_{1-42}$, and reaches its plateau after 6 h. The trend is consistent with that of ThT fluorescence assay, which further confirms that the Fc-KLVFF is capable of interacting with $A\beta_{1-42}$ instantaneously. In addition, a 40 μM Fc-KLVFF was incubated alone under the same experimental conditions as a control experiment. It was found that only 7.6% decrease in the anodic current of Fc-KLVFF occurred after 24 h of incubation in the mixed solvents, probably due to the slight aggregation of Fc-KLVFF. Consequently, the decrease of peak current of Fc-KLVFF in the presence of Aβ can be mainly attributed to the association of the Fc-KLVFF with $A\beta_{1-42}$ monomer and/or oligomer in solution to form insoluble adducts or the attachment of the Fc-KLVFF to $A\beta_{1-42}$ aggregates, but not the self-aggregation of Fc-KLVFF.

The major drawback for peptides as drugs in central nervous system diseases is their rapid degradation by proteolytic enzymes and poor membrane permeability. For example, the use of cholyl-LVFFA-OH as a therapeutic agent is restricted by its propensity to be cleared up almost completely after the hepatic first pass.[23] One of our aims of modifying of KLVFF with ferrocenoyl moiety is to improve its proteolytic stability in blood stream and increase

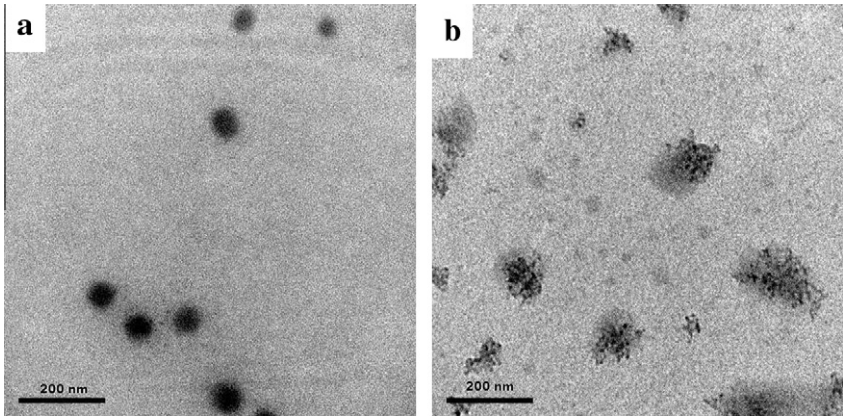


**Figure 5.** TEM images of 10 μM $A\beta_{1-42}$ incubated for 5 months at room temperature in the presence of 40 μM Fc-KLVFF (a), and 40 μM KLVFF (b).

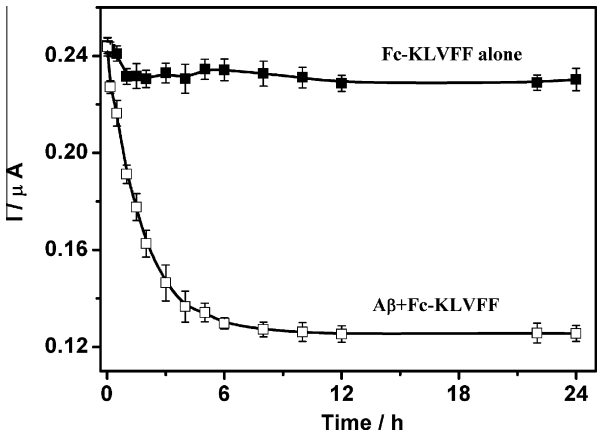


**Figure 6.** Inhibition of Fc-KLVFF towards $A\beta_{1-42}$ fibril formation detecting by using DPV method. The 40 μM Fc-KLVFF was incubated in the absence (■), and in the presence of freshly solubilized 10 μM $A\beta_{1-42}$ (□) in a mixed solvent of DMSO and water (3:1, v/v) at 32 °C for the indicated times. The experiment was repeated three times ($n$ = 3). Error bars represent the standard deviation (SD) of the current.

**Table 1**
Octanol/water partition coefficients for the compounds that were used in this study [a]

| Compound | log $k_w$ | log $P$ | Ref. |
|---|---|---|---|
| Fc-KLVFF (**5**) | 8.36 | 8.83 | This work |
| KLVFF (**6**) | 7.17 | 7.62 | This work |
| Ferrocene | — | 3.54 | [26] |

[a] log $P$ and log $k_w$ was determined by standard method.[17,25]

its lipophilicity. Proteolytic stability of KLVFF and Fc-KLVFF were determined by reverse-phase high-performance liquid chromatography (RP-HPLC). The experimental results showed that the modification with ferrocenoyl moiety increases the resistance of the compound to proteolysis. After 3 h incubation with proteolytic enzyme at 37 °C, only 18% of KLVFF was detected, while 58% of the Fc-KLVFF survived. After 24 h incubation, KLVFF was almost totally decomposed, while 48% of Fc-KLVFF still remained intact. The results are consistent with the experimental hypothesis, that is, the ferrocenoyl tethered KLVFF exhibits remarkable resistance to proteolytic degradation in comparison with its parent peptide KLVFF.

Lipophilicity is another important factor of drug permeability and plays a dominant role in toxicity predictions.[24] Here, the octanol/water partition coefficient (log $P$ value) was used to describe the lipophilicity of the inhibitors. To determine the lipophilicity of KLVFF and Fc-KLVFF, log $k_w$ values of all compounds were determined by RP-HPLC and then converted to log $P$ values by comparison to reference compounds[25] (cf. Table 1). The experimental results demonstrated that the substitution of a Boc group by a more lipophilic ferrocenoyl resulted in an increase in the lipophilic value of Fc-KLVFF compared to KLVFF itself.

In this Letter, aiming at improving the inhibitory effect, lipophilicity and stability towards proteolytic degradation of KLVFF, a potential inhibitor for Aβ aggregation, we tethered a redox and lipophilic ferrocenoyl moiety to pentapeptide KLVFF. The synthesized Fc-KLVFF showed high inhibitory effect upon Aβ fibril formation, good lipophilicity and high resistance to proteolytic degradation. In particular, the attachment of ferrocenoyl moiety onto peptides allows us to investigate the aggregation pathway of $A\beta_{1-42}$ in situ by electrochemical method. Nevertheless, the synthesized ferrocenoyl KLVFF shows poor solubility in water. In order to solve the problem, we are trying to synthesize the ferrocenoyl peptide with inclusion of some hydrophilic amino acid residues on the C-terminus, and planning to use this novel ferrocenoyl peptide to control neuronal toxicity from Aβ species as part of the future work in cellular system.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.07.111.

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