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Inhibitors of Fibril Formation and Cytotoxicity of β-Amyloid Peptide Composed of KLVFF Recognition Element and Flexible Hydrophilic Disrupting Element

Ken-ichi Watanabe,*,† Kazuhiko Nakamura,*,[1] Shingo Akikusa,*,† Tomoko Okada,* Masato Kodaka,* Takeo Konakahara,† and Hiroaki Okuno*

**National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan; and †Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*

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β-Amyloid peptide (Aβ) is the main protein components of neuritic plaques and its neurotoxicity would be exposed by formation of aggregate. The aggregation inhibitors composed of an Aβ recognition element (KLVFF) and a flexible hydrophilic disrupting element (aminoethoxy ethoxy acetate and aspartate) are designed and chemically synthesized. The inhibitory effects are examined by a pigment binding assay using Congo red or thioflavin T. The present compounds suppress the formation of aggregate, and the compound DDX3 is an especially effective inhibitor. In addition, the synthesized compounds efficiently suppress the cytotoxicity of Aβ against IMR-32 neuroblastoma cells *in vitro*. © 2002 Elsevier Science

β-Amyloid peptide (Aβ) consists of 39–42 amino acids, which is the major component of senile plaques in the brains of patients with Alzheimer's disease (AD) and known as a key substance for AD. Though the deposition of Aβ in the neural cells is reported as an early clinical phenomenon of AD, the molecular mechanism leading to AD remains to be elucidated (1–3). In an aqueous solution, Aβ forms aggregates spontaneously via peptide–peptide interactions. It was reported that Aβ itself is not toxic but it becomes toxic against neuronal cells once it is aggregated into amyloid fibrils (4, 5). Inhibition of the formation of amyloid fibrils has thus emerged as an approach to develop therapeutic drug for the treatment of AD. Tjerenberg and coworkers maintained that a short Aβ fragment (KLVFF, Aβ(16–20)) binds to full-length Aβ (6, 7). Recently, we developed a novel fluorescence assay for detecting molecular interactions by using various immobilized partial peptides of Aβ and a fluorescence probe possessing KLVFF (8), and as a result we concluded that the fluorescence-labeled KLVFF residue specifically recognizes the immobilized sequence KLVFF itself.

On the basis of our previous study, we designed some novel molecules consisting of a recognition element (KLVFF) and a hydrophilic disrupting element to inhibit molecular interaction between Aβ molecules. As a hydrophilic repeating unit, we chose aminoethoxy ethoxy acetic acid (AEEA) possessing ethylene glycol skeleton (Fig. 1). As AEEA can be connected repetitively, the hydrophilicity of an inhibitor can be controlled by changing the number of AEEA units. This type of inhibitor is termed Xn (n = 1–6) in the present study. To add more hydrophilicity to the disrupting element, we further designed hybrid-type derivatives composed of AEEA and aspartate (D) (Fig. 1). We selected aspartate because it is the simplest amino acid with a negatively charged side chain in biological conditions and thus we expected it to elevate the ability of disrupting elements by binding more water around them. We named this type of inhibitors DDXn (n = 1–3), though in the present study only DDX3 was investigated in detail because of the poor solubility of DDX1 and DDX2. These inhibitors were synthesized in a solid-phase manner using a Pioneer peptide synthesizer (Applied Biosystems, Inc.) by Fmoc chemistry.

Furthermore, we examined fibril formation of Aβ (Aβ 1-40) using a pigment-binding method, in which Congo red (CR) (9, 10) or thioflavin T (ThT) (11) was used to measure the fibril concentration. The inhibitory assay of Aβ neurotoxicity was also carried out using IMR-32 neuroblastoma cells *in vitro*.

MATERIALS AND METHODS

Synthesis of KLVFF-related peptides. The synthesis of KLVFF-related peptides was started with PAC-PEG-PS resin (Applied Bio-

[1] To whom correspondence and reprint requests should be addressed. Fax: +81-298-61-6123. E-mail: kazuhiko.nakamura@aist.go.jp.

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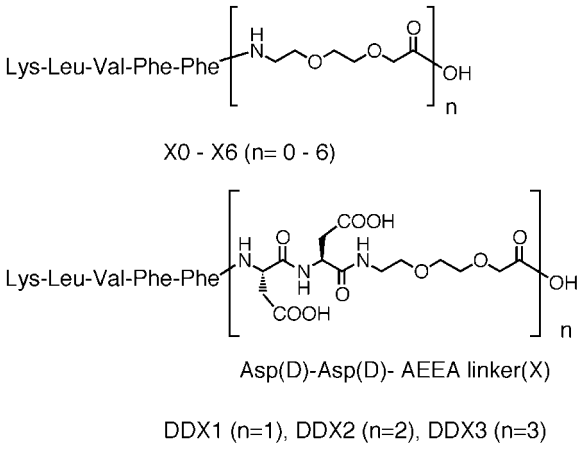

FIG. 1. Structures of aggregation inhibitor.

systems, Inc.) by loading Fmoc-AEEA (Applied Biosystems, Inc.) with DCC-DMAP. For the compound without AEEA (X0), phenylalanine-preloaded Wang resin was selected for the starting solid-phase. The peptide elongation was performed by repeating condensation of Fmoc amino acids by HATU (*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′*N*′-tetramethyluronium hexafluorophosphate) chemistry using a Pioneer peptide synthesizer. The final cleavage from the resin was accomplished by TFA cleavage mixture (TFA:TIS:H_2O = 95:2.5:2.5). The crude products were purified with HPLC on an ODS column using an acetonitrile–water gradient. The purity of final compounds was verified by analytical HPLC, and the identification was assessed by correct MS.

MALDI-TOF MS data for synthesized compounds: X0: calcd. 653.8 (MH^+), found 653.8; X1: calcd. 798.9 (MH^+), found 798.9; X2: calcd. 944.1 (MH^+), found 943.8; X3: calcd. 1089.3 (MH^+), found 1089.3; X4: calcd. 1234.4 (MH^+), found 1234.1; X5: calcd. 1379.6 (MH^+), found 1379.2; X6: calcd. 1524.7 (MH^+), found 1524.5.

DDX1: calcd. 1028.2 (MH^+), found 1028.2; DDX2: calcd 1419.5 (MH^+), found 1420.0; DDX3: calcd. 1778.7 (MH^+), found 1778.5.

Pigment binding assay Aβ was dissolved in DMSO (1 mM), and the solution was immediately added to the solution (Milli-Q water) of the inhibitor so that the final concentrations were 100 μM for Aβ and 200 μM for the inhibitor. After incubation at 37°C for a given time, Aβ solution (control sample) (5 μL) or Aβ-inhibitor mixture solution (5 μL) was individually added to 120 μL of PBS (pH 7.2) solution containing Congo red (25 μM). After 2 h, absorbance at 540 nm (A_{540}) and 480 nm (A_{480}) was measured on a Shimadzu CS-9300PC plate reader, and fibril concentration (F) was calculated by the following equation (9, 10):

$$F = A_{540}/25295 - A_{480}/46306. \quad [1]$$

To further confirm the fibril formation, we also determined the fibril concentration by the ThT fluorescence method (11). Aβ solution (control sample) (5 μL) or Aβ-inhibitor mixture solution (5 μL) was individually added to 400 μL of PBS (pH 7.2) buffer solution containing ThT (10 μM), and fluorescence intensities were measured (λ_{ex} = 450 nm, λ_{em} = 482 nm) with a JASCO FP-777 spectrofluorometer.

Neurotoxicity assay. The tetrazolium-based semiautomated colorimetric assay (MTT assay) (12) was modified and used for the neurocytotoxicity assay. IMR-32 cells (1×10^6/well, 180 μL/well) in culture medium were seeded in a 96-well flat-bottom microplate, and 20 μL of Aβ solution (with or without the inhibitor) was added to each well. The final concentration of Aβ and the aggregation inhibitor was 0.5 and 1.0 μM, respectively. The plate was incubated for 1 day at 37°C in 5% CO_2, and the MTT reagent (prepared at a concentration of 5 mg/mL in PBS) was added to each well (10 μl/well). After another incubation for 4 h at 37°C, to solubilize the resulting MTT–formazan, 100 μL/well of 20% SDS in deionized water and DMSO was added to each well followed by thorough mixing with a mechanical plate mixer. Absorbance at 540 nm was measured on a Shimadzu CS-9300PC plate reader.

RESULTS AND DISCUSSION

Effects of Xn (n = 0–6) inhibitors on the fibril formation are illustrated in Fig. 2. Without any inhibitors, fibril formation of Aβ can be observed after 3–4 days under the physiological condition. In our experiments, on day 4, fibril formation began only in the inhibitor-free control sample. On days 5 and 6, the inhibitory effect is well correlated with the number (n) of AEEA units in the disrupting element; that is, the longer disrupting elements tend to have the higher inhibitory activity. On day 7, the dependency on the length of the disrupting element is almost lost, though the inhibitory activities remain to some extent. These facts clearly indicate that this class of compounds inhibits the growth of fibrils.

More prominent influence is observed by the hybrid-type inhibitor, DDX3 (Fig. 3). Similarly to Xn inhibitors, fibril formation is scarcely observed when the incubation time is less than 4 days. In contrast to Xn inhibitors, however, no fibril formation is observed up to day 7 in the presence of DDX3. This result suggests that Aβ is isolated in solution by the formation of a stable complex with DDX3 without interaction to Aβ itself. The molecular design of an inhibitor of aggregation of Aβ has thus been successfully accomplished through our experiments.

Though a similar concept was recently reported by Lowe *et al.* (13), they used natural amino acid residues with a negative, positive or neutral charge (*viz.*, glutamate (E), lysine (K), serine (S)) as repeating units of disrupting elements. In contrast to their inhibitors, we introduced an unnatural ω-amino acid when designing the disrupting elements. Using another approach, Gordon *et al.* synthesized N^{α} methylated KLVFF containing peptides as fibrillogenesis inhibitors (14). One of the characteristics of our novel inhibitors is that one can freely control physicochemical properties such as conformational flexibility, charges and hydrophilicity. By choosing AEEA linker, conformational flexibilities can be efficiently realized. The advantages of AEEA linker in high solubility and biological inactiveness are due to the structural properties of the repeating unit. Our fundamental strategy is that there is no reason to restrict the repeating units to natural amino acids and that hydrophilic repeating units should be as simple as possible.

While examining the inhibitory effect on fibril formation, we evaluated the effect of Xn and DDX3 on the

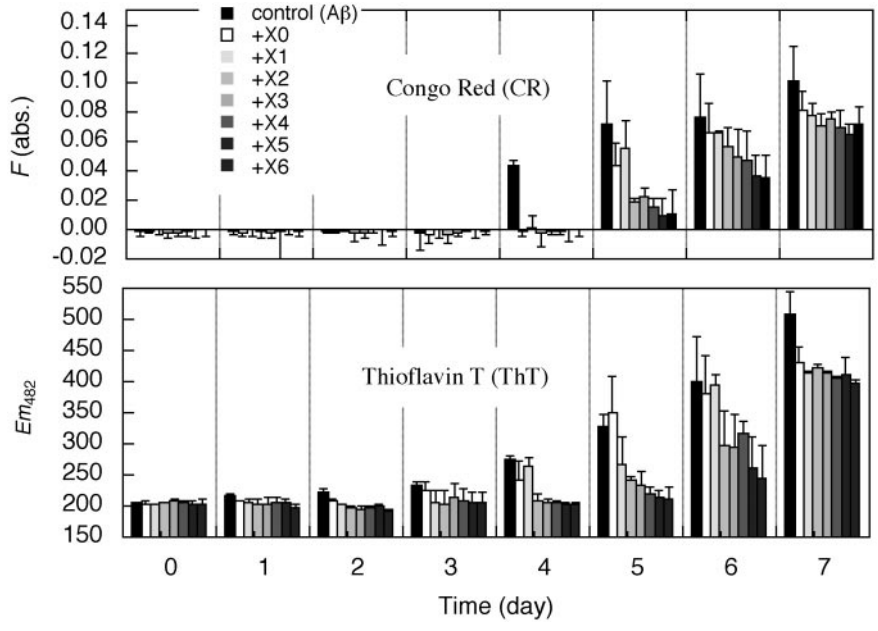

FIG. 2. Fibril formation of Aβ.

cytotoxicity of Aβ against human neurofibroblast IMR-32 cells. The Xn inhibitors as a whole tend to prevent Aβ cytotoxicity (Fig. 4), while remarkable dependency of cell viability on the number (n) of the repeating units of AEEA is observed on days 3 and 4. As predicted from the above inhibitory experiment of fibril formation, DDX3 is very effective at inhibiting Aβ toxicity as shown in Fig. 4, where the cell viability reaches almost 100% in the presence of DDX3. Therefore, we concluded that the detoxication of Aβ with these compounds is due to the inhibition of fibril formation.

One difference between our inhibitors (Xn and DDX3) and the inhibitors reported by others (KLVFF-S4, KLVFF-K4 and KLVFF-E4) (13) is that Xn inhibitors prevent both fibril formation and cytotoxicity of Aβ but KLVFF-S4 had no inhibitory effects, though neither of these compounds has a charge in the disrupt-

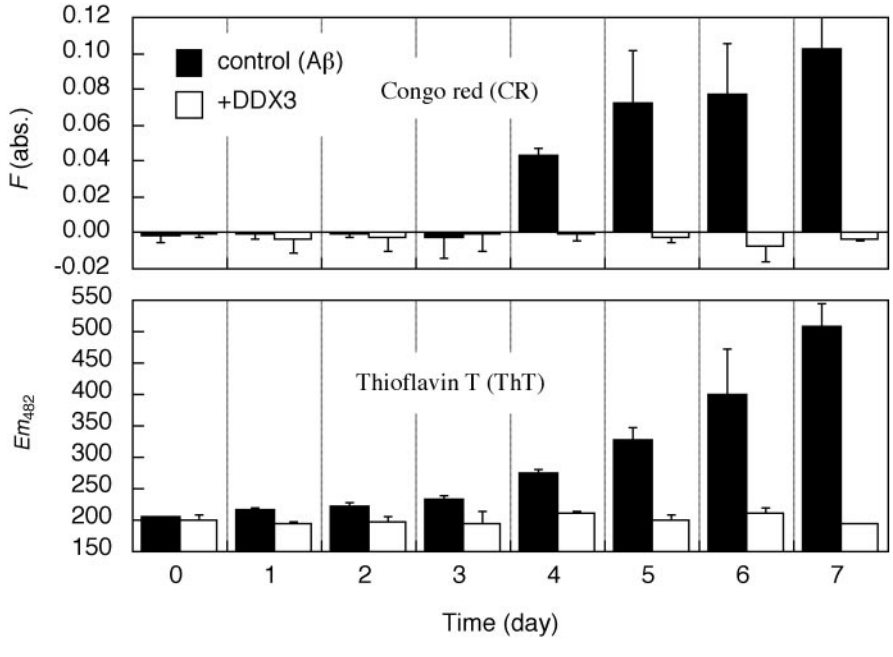

FIG. 3. Inhibition of Aβ aggregation with DDX3.

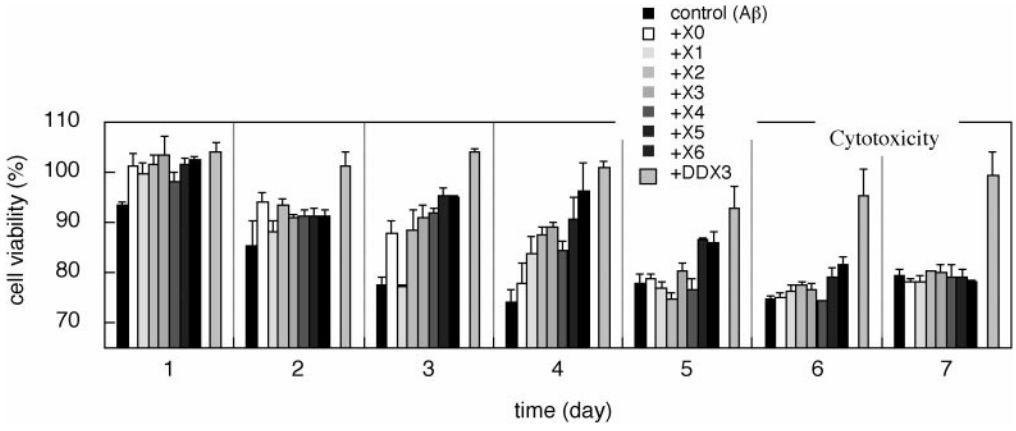

FIG. 4. Detoxication of Aβ to IMR-32 neuroblastoma cells.

ing elements. The second distinct difference is that KLVFF-K4 and KLVFF-E4 accelerate the aggregation but have the cytotoxicity, whereas DDX3 inhibits both fibril formation and cytotoxicity, though all these compounds have negative or positive charges in the disrupting element. The fundamental structure of AEEA, the ethylene glycol unit, is thought to make the disrupting elements flexible compared to peptide type repeating units. This conformational flexibility is related to these differences in inhibitory effects, whereas the contribution resulting from the difference in hydration cannot be neglected. Namely, these two factors (conformational flexibility and degree of hydration) may be necessary to evaluate the disrupting elements, both of which tend to prevent the interaction between Aβ molecules.

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