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Different inhibitory response of cyanidin and methylene blue for filament formation of tau microtubule-binding domain

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ABSTRACT

One of the priorities in Alzheimer research is to develop a compound that inhibits the filament formation of tau protein. Since the three- or four-repeat microtubule-binding domain (MBD) in tau protein plays an essential role in filament formation, the inhibitory behavior of cyanidin (Cy) and methylene blue (MB) with respect to heparin-induced filament formation of MBD in a neutral solution (pH 7.6) was characterized by fluorescence, circular dichroism, and electron microscopy measurements. The planar aromatic ring of Cy and the N-unsubstituted phenothiazine ring of MB were shown to be necessary for the inhibition. However, the inhibitory responses with respect to heparin-induced filament formation to the second and third repeat peptides of MBD were different: Cy suppresses the formation and MB does not prevent the formation. This suggests the importance of the first and fourth repeat peptides in the inhibitory activity of MB for MBD filament formation. In this study, we showed that the decrease of thioflavin S fluorescence intensity is not always linked to inhibition of filament formation.

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population. AD is characterized by two histopathological hallmarks: extracellular deposits of β-amyloid in neuritic plaques, and intracellular neurofibrillary tangles (NFTs) [1]. The latter are composed of bundles of paired helical filaments (PHFs), caused by the abnormal aggregation of tau protein. Tau is a neuronal microtubule-associated protein (MAP), and its important function is the stabilization of axonal microtubules [2]. Tau is a highly soluble protein and rarely shows any tendency to assemble under physiological conditions. In the brains of AD patients, however, it dissociates from its natural partner, the microtubule, and aggregates to form insoluble fibers [3]. Therefore, one of the priorities in Alzheimer research is to develop a compound that is able to inhibit PHF formation by tau protein.

Tau protein includes the microtubule-binding domain (MBD) of three or four imperfect 31- to 32-residue repeats (3RMBD or 4RMBD, respectively, Fig. 1) [3]. As the MBD located in the C-terminal half constitutes the core of the tau filament [4–6] and is capable of self-assembling into a filament similar to that of full-length tau *in vitro* [7], it is reasonable to investigate the effect of inhibitors on tau filament formation using 3RMBD, 4RMBD and each repeat peptide.

Several molecules capable of both inhibiting aggregation and promoting filament disaggregation of tau protein have been reported [8–11], but knowledge of the structure-based inhibitory mechanism is fragmentary and imperfect. Cyanidin (Cy) and methylene blue (MB) are representative inhibitors of polyphenol and phenothiazine classes, respectively [11]. To investigate the relationship between their chemical structures and inhibitory modes in greater detail, we investigated the inhibitory behavior toward heparin-induced filament formation of MBD and its repeat peptides by thioflavin S (ThS) fluorescence, circular dichroism (CD) and electron microscopy (EM) measurements in a neutral solution. In this paper, we report the different responses of Cy and MB with respect to self-aggregations of the second (R2) and third (R3) repeat peptides; these two compounds exhibited similar inhibitory effects on filament formations of 3RMBD and 4RMBD. The schematic of full-length tau, 4RMBD, and 3RMBD, and the chemical structures of Cy, MB, and their related compounds used in this work are shown in Fig. 1.

Materials and methods

Chemicals and recombinant proteins. Cy chloride, MB hydrate, heparin (average molecular weight = 6000 Da), and ThS were

Abbreviations: AD, Alzheimer's disease; CD, circular dichroism; Cy, cyanidin; His, histidine; EM, electron microscopy; MAP, microtubule-associated protein; MB, methylene blue; MBD, microtubule-binding domain; 3RMBD, three-repeated MBD; 4RMBD, four-repeated MBD; MT, microtubule; NFT, intracellular neurofibrillary tangle; PHF, paired helical filament; ThS, thioflavin S.

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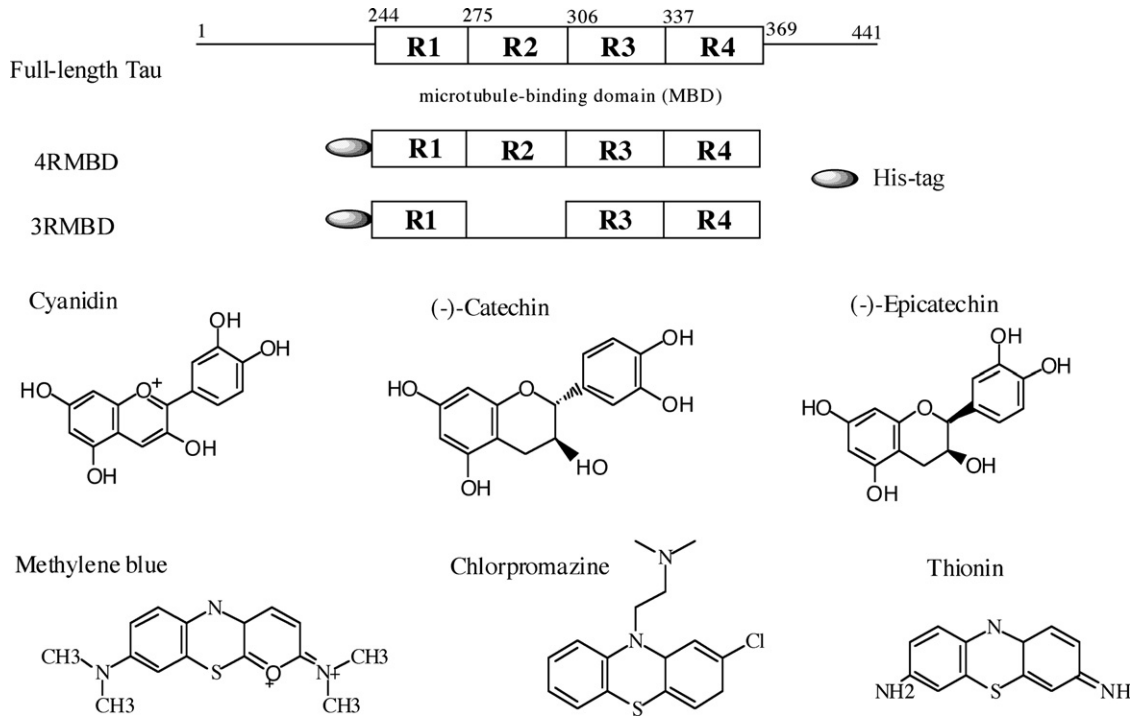

Fig. 1. Schematic of tau protein including four-repeat microtubule-binding domain and His-tagged 4RMBD and 3RMBD, and chemical structures of cyanidin, methylene blue, and their analogs used in this work. The regions from the first to the fourth repeat fragments in 4RMBD are named R1 to R4, respectively. The numbering of the amino acid residues in entire tau protein refers to the longest isoform of the human tau protein (441 residues).

obtained from Sigma (St. Louis, USA). Repeat peptides (R1–R4) were synthesized using a solid-phase peptide synthesizer. The peptides were characterized by mass spectrometry and had a purity of >95.0% as assessed by reverse-phase high-pressure liquid chromatography. The samples were obtained as lyophilized powders.

The gene expressions and purifications of the recombinant His-tagged 3RMBD and 4RMBD (human brain) were performed as described in the literature [12]. Their purities were confirmed as single bands under reducing conditions by sodium dodecylsulfate–polyacrylamide gel electrophoresis.

CD measurement. Solutions of 25 μM MBD or repeat peptide in the absence or presence of inhibitor (1–50 μM) were prepared with buffer A (50 mM Tris–HCl buffer (pH 7.6)) and mixed with heparin (6.25 μM). All measurements were conducted at 25 °C with a JASCO J-820 spectrometer in a cuvette with a 0.5 mm path length. For each experiment under N_2 gas flow, the measurements from 190 to 250 nm were repeated eight times and summed. Then, molar ellipticity was determined after normalizing the peptide concentration. The same experiments were performed three times using freshly prepared samples. Data are expressed in terms of means residue ellipticity [θ] in units of deg cm^2 dmol^{-1}.

ThS fluorescence. The concentration of MBD or repeat peptide of 25 μM in the absence or presence of inhibitor (1–50 μM) was adjusted with buffer A containing 10 μM ThS. Heparin (final concentration = 6.25 μM) was then added to the solution. The fluorescence intensity was measured on a JASCO FP-770F instrument with a 2-mm quartz cell maintained at 25 °C using a circulating water bath. The kinetics of the spectral change of MBD or repeat peptide were analyzed by recording the time-dependent profile of the fluorescence with an excitation at 440 nm and an emission at 500 nm. The background fluorescence of the sample was subtracted when necessary.

Electron microscopy (EM). The concentration of each MBD or repeat peptide of 25 μM in the absence and presence of inhibitor (50 μM) was adjusted with buffer A containing 6.25 μM heparin. The solution was then incubated at 25 °C for 48 h. The 600-mesh copper grids were used for negative-staining EM. One drop each of the protein solution and 2% uranyl acetate was placed on the grids. After 1 min, excess fluid was removed from the grids. Negative-staining EM was performed using an electron microscope (Hitachi Co.) operating at 75 kV.

Results and discussion

Inhibition of filament formation of MBD by Cy and MB

Both Cy and MB inhibited the heparin-induced filament formation of 4RMBD and 3RMBD in a concentration-dependent manner; the quantities of MBD filaments, estimated from EM pictures, decreased in proportion to the decreased ThS fluorescence intensities (Fig. 2A and B), although the IC_{50} values of both compounds (Table 1) were considerably different compared with their inhibition rates of filament formation in EM images. As it has already been reported that MB and analogs of Cy inhibit full-length tau protein formation [11], this result suggests that the major interaction region of these inhibitors is in the repeated MBD. The inhibitory effect on the filament formation of MBD was commonly observed with both inhibitors. They inhibited the filament formation of 4RMBD more efficiently than 3RMBD and formed sphere-like aggregates more frequently in 3RMBD than 4RMBD; such granular tau oligomers have been considered intermediates of tau protein en route to the filament formation [13]. The result indicates that the different inhibitory effect of both inhibitors on 4RMBD and 3RMBD is primarily due to the presence or absence of the R2 repeat moiety.

To investigate the effect of Cy and MB on the heparin-induced conformational transition of 4RMBD, the time-dependent CD spectral changes were recorded in the absence and presence of inhibitor (Fig. 3A and Table 1); similar changes were also observed for 3RMBD (data not shown). Consequently, it was clarified that both Cy and MB inhibit the conformational transition of 4RMBD to nearly the same

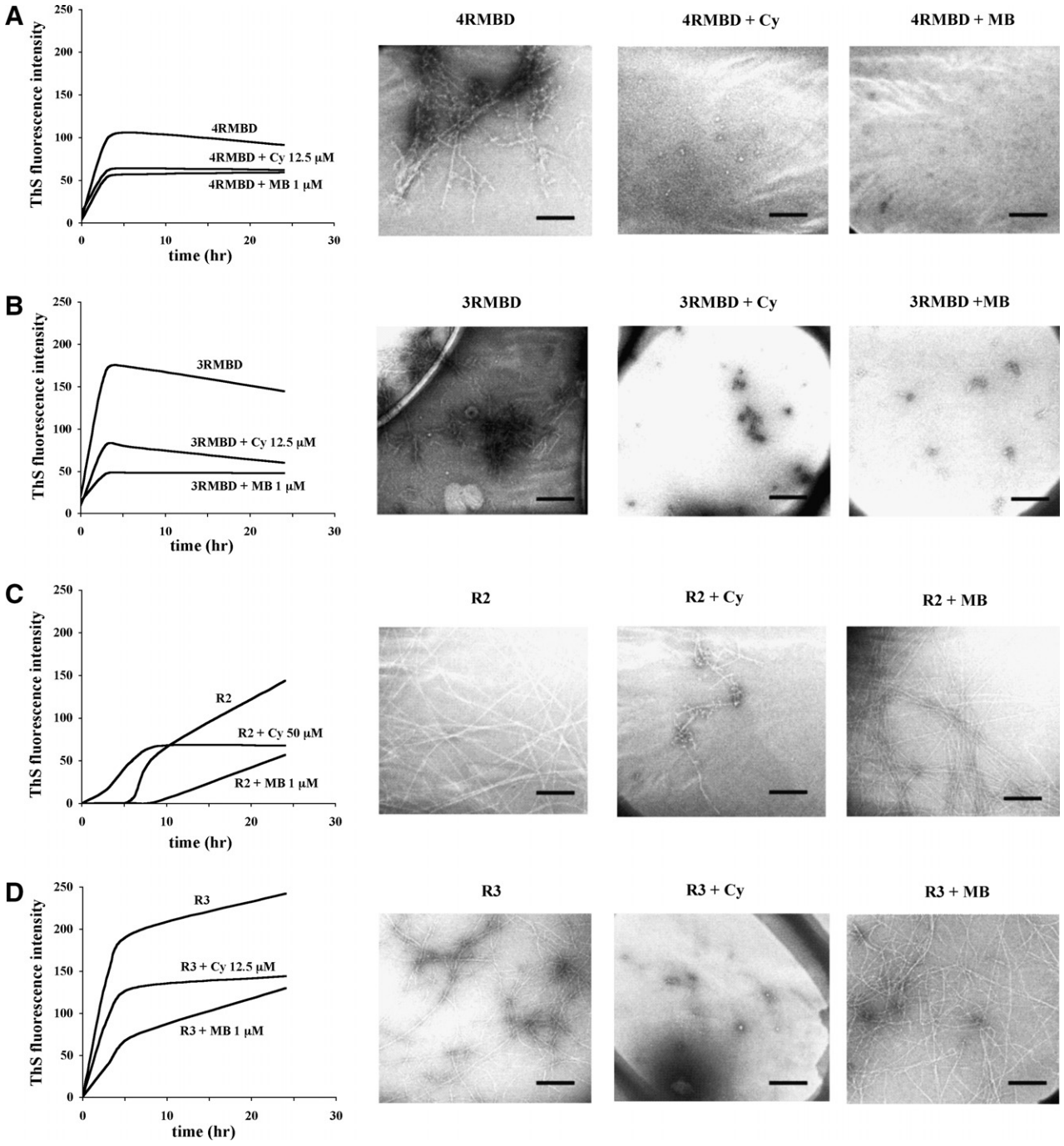

Fig. 2. Time vs. ThS fluorescence intensity profiles (left side) and EM filaments of heparin-induced filament formation (right side) of (A) 4RMBD, (B) 3RMBD, (C) R2, and (D) R3 (25 μM in each) in the absence and presence of Cy (12.5 or 50 μM) or MB (1 μM) in 50 mM Tris–HCl buffer (pH 7.6). Bar = 400 nm.

Table 1
Parameters for defining characteristics of time-scanned ThS fluorescence and CD profiles of 4RMBD, 3RMBD, R2, and R3 repeat peptides (25 μM)

Repeat peptide inhibitor	IC_{50} value by ThS filament assay[a]		Conformational transition rate by CD ($[|\Delta\theta|]/h$)[b,c]		
	Cy (μM)	MB (μM)	control	Cy (25 μM)	MB (25 μM)
4RMBD	25	1	420	100	110
3RMBD	12	0.3	700	350	160
R2	50	0.4	800	390	520
R3	25	1	4600	1700	3400

[a] 50% decreasing concentration of ThS fluorescence intensity.
[b] Absolute change in the value of molar ellipticity $|[\theta]|$ at 198 nm per unit (h).
[c] The variation is in the range of ±15%.

extent. However, the inhibition of MB for the transition of 3RMBD was more effective than that of Cy, indicating the stabilization of the 3RMBD random structure by MB; this may relate to the lower IC_{50} values of MB in the ThS filament assay. The CD results suggest that the inhibition of filament formation by Cy and MB is mainly due to the stabilization of MBD random structure.

Structural requirements of Cy and MB for inhibition of MBD filament formation

In order to examine the structural requirements of Cy and MB for inhibiting the filament formation of MBD, the inhibitory effects

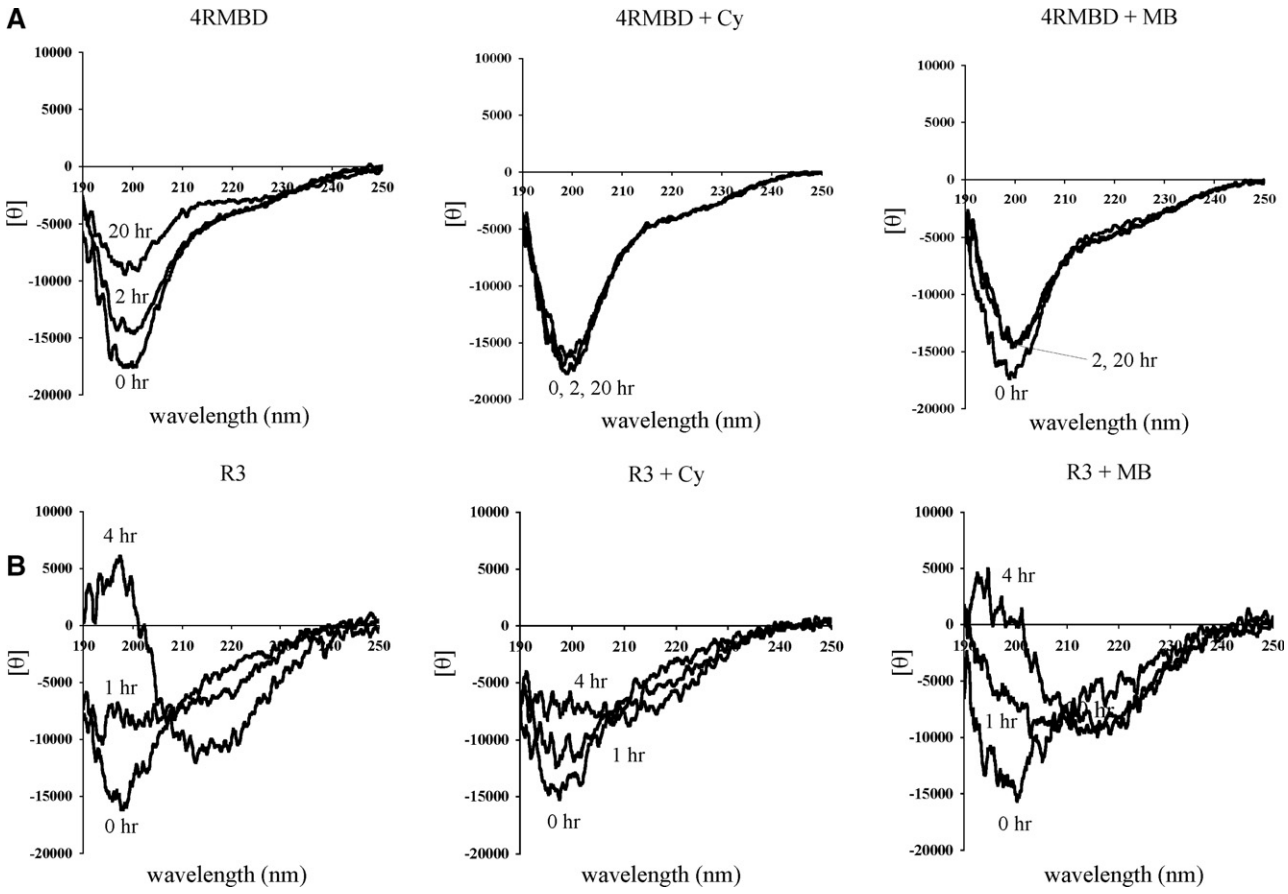

Fig. 3. CD profiles as a function of time of heparin-induced conformational changes of (A) 4RMBD and (B) R3 (25 μM in each) in the absence and presence of Cy or MB (25 μM) in 50 mM Tris–HCl buffer (pH 7.6).

of structurally related compounds were measured using the ThS fluorescence assay. The results are shown in Fig. 4. Compared with Cy or MB-treated 3RMBD and 4RMBD, the inhibiting effects of these compounds were considerably lower, indicating their poor inhibitory abilities. Because EM measurements also gave similar results, we suggest that the N-unsubstituted phenothiazine ring of MB and the planar anthocyanidin ring of Cy are important for inhibitory activity: the importance of the N-unsubstituted phenothiazine ring has also been reported by Taniguchi et al. [11].

Different responses to filament formation by the second and third repeat peptides with Cy and MB

To analyze the inhibitory features for MBD with Cy and MB in more detail, their behavior toward filament formation by each repeat peptide in MBD was examined; filaments of R1 and R4 repeat peptides were not formed in the absence or presence of inhibitors because of their lack of self-aggregation ability in neutral solution [14]. The parameters for characterizing the inhibitor-dependent

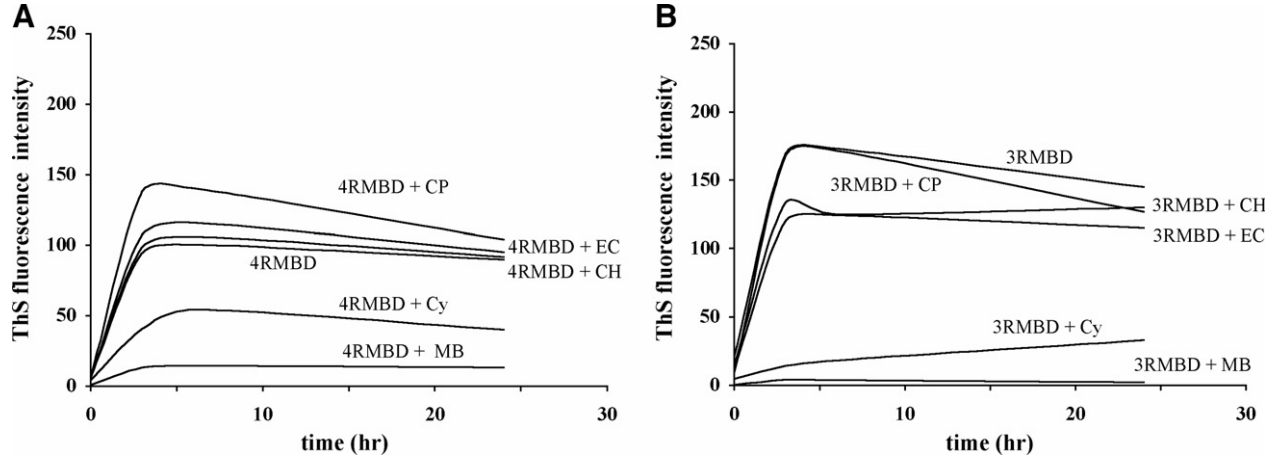

Fig. 4. ThS fluorescence intensity changes of (A) 4RMBD and (B) 3RMBD (25 μM) in the presence of chlorpromazine (CP) and MB (12.5 μM in each), and (−)-cathechin (CH), (−)-epicatechin (EC), and Cy (25 μM in each) in 50 mM Tris–HCl buffer (pH 7.6).

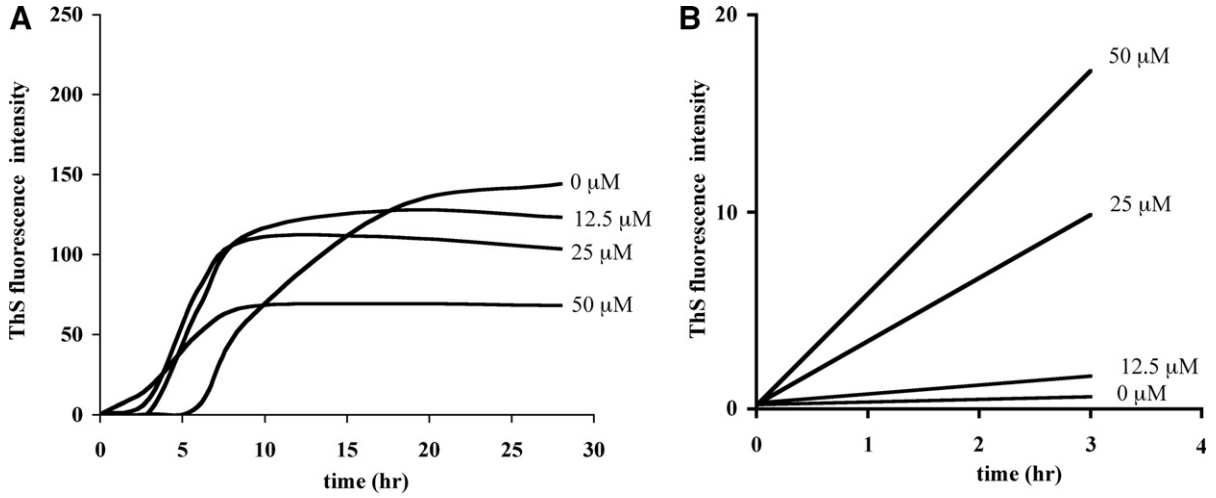

Fig. 5. (A) Cy concentration-dependent change of ThS fluorescence intensity as a function of time and (B) linear increase of ThS fluorescence intensity for 0–3 h after adding heparin into R2 solution (25 μM) in 50 mM Tris–HCl buffer (pH 7.6). The concentration of Cy is given on each line.

ThS fluorescence and CD spectra of R2 and R3 peptides are given in Table 1.

The ThS fluorescence and EM measurements (Fig. 2C and D) show that Cy inhibits filament formation by R3, and the inhibition profile is similar to those of 3RMBD and 4RMBD. In contrast, a different behavior was observed for the fluorescence profile of R2. Cy accelerated the self-aggregation of R2 in proportion to the concentration of Cy (Fig. 5) for several hours after the start of the reaction, although the EM measurements showed that Cy ultimately inhibits the filament formation relative to inhibitor-free R2. As R2 has a lag time of ~4 h before self-aggregating in 50 mM Tris–HCl buffer (pH 7.6) [14], Cy appears to have sufficient time to interact with R2, consequently accelerating the self-aggregation time of R2, although this behavior does not lead to the increae of filament formation. In the case of R3, however, association with Cy appears not to affect self-aggregation because of the fast self-aggregation behavior of R3 (lag time ~ 0 h).

On the other hand, MB did not inhibit filament formation of R2 and R3, although it caused a prominent decrease in ThS fluorescence intensity (Fig. 2C and D); thionin (Fig. 1) showed similar behavior. Generally, it is now accepted that the increased intensity of ThS fluorescence is proportional to the extent of filament formation [15]. However, our results indicate that a decrease in ThS fluorescence intensity does not always reflect inhibition of filament formation. Although the exact reason why MB and thionin exhibit such unusual behavior is unclear, the following may be considered: the binding of MB to the heparin-induced conformation of R2 and R3 interferes with the binding of ThS. In this situation, a decrease in ThS fluorescence intensity would be caused by the competitive binding between MB and ThS with respect to R2 or R3 peptide. This situation may not arise for Cy, because it significantly inhibits the heparin-induced conformational transition of R2 and R3 (Fig. 3B and Table 1), indicating a different mode of binding of Cy and MB to the peptide.

Importance of R1 and R4 peptides for inhibition of MBD filament formation

Although Cy and MB are known as inhibitors representative of polyphenol and phenothiazine families, respectively, the different response of R2 and R3 peptides to them was shown for the first time in this study, i.e., the inhibition by Cy but no effect of MB on filament formation by R2 and R3 peptides. Nevertheless, MB exhibited concentration-dependent inhibition of filament formation by 3RMBD and 4RMBD. This indicates the important contribution of R1 and R4 repeats to the inhibition (Fig. 1). As R1 and R4 peptides themselves have no ability to form heparin-induced self-aggregates in a neutral solution [14], their behavior of promoting the inhibition of MBD filament formation is remarkable. Our results indicate the supportive role of R1 and R4 in the inhibition of MB for filament formations by 3RMBD and 4RMBD, and this result contrasts with the negative role of R2 and R3. Concerning this aspect, it is noteworthy that our recent study clarified the function of R1 and R4 repeats as repressors or modifiers of filament formation and the topology of MBD [16].

In contrast, the concentration-dependent inhibition of Cy was observed in both the repeat peptides and MBD, where the decreased intensity of ThS fluorescence paralleled the degree of inhibition of filament formation. It is obvious that such characteristic differences between MB and Cy in terms of the inhibitory response are primarily due to their different chemical structures. Thus, these results may be useful for developing a repeat peptide-specific low molecular-weight inhibitor for PHF formation in Alzheimer's disease.

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