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Inhibition by Aplidine of the aggregation of the prion peptide PrP 106–126 into β-sheet fibrils

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Abstract

Aplidine, a cyclic peptide, from the tunicate *Aplidium albican*, prevents the in vitro aggregation into β-sheet containing fibrils of the prion peptide 106–126 when co-incubated in a 1:1 molar ratio. The blocking of fibril formation induced by Aplidine has clear sequence specificity, being much stronger for the 106–126 prion peptide than for the β-amyloid 25–35 peptide. In addition to the known ability of Aplidine to cross the plasmatic membrane, these results indicate that Aplidine is a potential leading compound for the development of therapeutic blockers of prion aggregation.

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Keywords: Aplidine; Prion peptide; Inhibition of aggregation

1. Introduction

Prion diseases are a group of typically fatal neurodegenerative diseases clinically characterized by the appearance of spongiform lesions in the central nervous system (CNS). Members of this group are diseases affecting humans: Creutzfeldt–Jakob disease, fatal familial insomnia, Gerstmann–Straussler–Scheinker disease and kuru, as well as others affecting animals, such as scrapie and bovine spongiform encephalopathy [1]. The manifestation of the disorder can have a genetic origin, arise sporadically, or even be caused by infectious transmission [2]. In all these cases, the disorder is accompanied by a structural modification of the cellular, primarily α-helical, prion protein (PrP^C) into an aggregated and protease-resistant form that displays a high β-sheet content (PrP^{sc}) [3–5].

The prion hypothesis, which was distinguished with the Nobel Prize to Stanley Prusiner in 1997, states that it is the change in protein conformation from PrP^C to PrP^{sc} which causes the disease and transforms the cellular PrP protein into a novel class of infectious particle. The definitive proof for the prion hypothesis, namely the chemical transformation of normal PrP protein into infectious particles, has not been achieved yet. However, during the last two decades, a large body of evidence supporting this hypothesis has been assembled [6]. Clinical correlations have been found between the onset of the disease and the appearance of PrP^{sc} deposits in brain tissue [7,8]. The infectious particles are mainly composed of the same aggregates of PrP in an abnormal conformation, and do not include significantly sized nucleic acids [3,9]. A simple protein-only mechanism of replication and transmission has been proposed that involves recruitment of normal PrP protein to form new infectious particles [3,6]. Genetic modified mice experiments reveal that expression of PrP^C protein is, indeed, required for the onset of the disease [10].

However, only a fraction of the prion protein seems to be involved in all these processes. The major component extracted from amyloid inclusions of GSS brain tissue is a 7 kDa fragment comprising residues 88–153 of the prion protein [11]. A smaller fragment comprising residues 90–144 appears to be able to initiate the prion disease in vivo when it carries the P102L substitution (a GSS-linked mutation) [12]. Furthermore, the region involved in the conformational change only encompasses residues 106–

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141 of the PrP sequence. In the native structure, this region includes two short β-strands [1] that are thought to elongate upon conversion into PrP^{sc}. Peptides corresponding to segments of the 106–141 region aggregate forming β-structure in vitro [13], suggesting that the conformational change can be replicated without the context of the whole protein. One of such peptides—i.e. peptide 106–126—has been shown to kill neuronal cells in culture after formation of extracellular β-sheet-containing aggregates [3,14,15]. Furthermore, the formation of β-sheet structure in peptide 106–126 requires a pH of ~ 5, in agreement with the hypothesis that the transformation into PrP^{sc} occurs in the lysosomes [1,16].

For these reasons, region 106–141 of PrP has often been used as a target for developing and testing molecules with potential therapeutic activity against PrP^{sc}. Peptides from this region inhibit the in vitro incorporation of recombinant PrP into PrP^{sc} particles [17], probably by sequestering PrP into peptide aggregates. In a more recent report, it was shown that peptides originating from this region and modified with several prolines to act as β-sheet breakers were capable of dissolving infectious PrP^{sc} [18]. Moreover, co-incubation of PrP^{sc} with these β-breaker peptides decreased the infectivity of PrP^{sc} and delayed the onset of clinical symptoms of scrapie in animal models [18].

In this report, we take a different approach. Instead of using analogs of the PrP sequence as a base for finding possible inhibitors of PrP aggregation, we look for more generic drugs known to act as β-breakers. In particular, we have tested the inhibitory effect of the marine drug Aplidine [7], a proline-containing cyclic peptide isolated from the tunicate *Aplidium albican* [19], on the in vitro aggregation of peptide PrP 106–126 into β-sheet fibrils. We find that Aplidine is a strong inhibitor of the aggregation of PrP 106–126, showing measurable effects even at ~ equimolecular concentrations. Moreover, the effect of Aplidine on the aggregation of the β-amyloid peptide, another β-sheet fibril-forming peptide [20], is much less pronounced, indicating specificity of action against the PrP 106–126 sequence.

2. Materials and methods

2.1. Materials

Aplidine was provided by PharmaMar (Madrid, Spain).

2.2. Assembly of peptides

In vitro polymerization experiments of PrP 106–126, and β-amyloid 25–35 peptides have been performed as previously indicated [14,15,21]. Briefly, filaments were grown by vapor diffusion in hanging drops in the standard way used for protein crystallization [22]. Ten micrograms of PrP 106–126 (1 mg/ml final concentration), or increasing amounts from 0 to 5 μg of β-amyloid 25–35 peptide (concentrations between 0 and 0.5 mg/ml) were dissolved in 10 μl of Buffer A (0.1 M MES (pH 6.4), 0.5 mM $MgCl_2$ and 2 mM EGTA) plus 50 mM NaCl. The reservoir contained 0.2 M NaCl in buffer A. The hanging drops were incubated for 4 days at 4 °C.

2.3. Effect of Aplidine in peptide assembly

Tests of the inhibitory effect of Aplidine in peptide assembly were performed by adding Aplidine and the PrP or β-amyloid peptides simultaneously to the same incubation buffer described above. The hanging drops were incubated for 4 days at 4 °C. Tests of the effect of Aplidine in preformed fibrils of PrP peptide were carried by adding Aplidine at a final concentration of 500 μM to samples containing fibrils assembled by the standard hanging drop procedure (as explained in the previous paragraph). The samples were incubated with Aplidine up to 2 h at room temperature, and then inspected by electron microscopy.

2.4. Fourier transformed infrared (FTIR) spectroscopy

All the infrared absorption spectra were obtained in aqueous solution using a Digilab Excalibur FTS 3000 FTIR spectrometer equipped with a Harrick ConcentratIR horizontal multiple reflection (ATR) [23]. This accessory is specially designed to carry out FTIR measurements of very small volumes of liquid by producing 14 infrared reflections within a 4-mm diameter area. For each spectrum, 512 interferograms at 4 cm^{-1} resolution were co-added, and Fourier-transformed using triangular apodization. Samples of PrP 106–126 were prepared by dissolving 0.25 mg of peptide in 25 μl of PBS buffer plus either 5 μl of DMSO, or 5 μl of a 200 mg/ml Aplidine solution in DMSO. The samples were incubated overnight at 37 °C, then freeze-dried and resuspended in 30 μl of D_2O to carry out the FTIR measurements. β-Amyloid 25–35 samples were prepared exactly in the same way, but using water instead of PBS buffer.

3. Results

Fig. 1A shows a negative-staining electron micrograph of a sample of ~ 430 μM PrP 106–126 in conditions known to induce aggregation into β-sheet containing fibrils (see Materials and methods). The image shows large amounts of long fibrillar aggregates that tangle up into a rather dense mesh. The overall dimensions of these filaments, with a diameter of 6–8 nm, correspond to those previously described for PrP 106–126 [15]. Addition of Aplidine in a 1:9 ratio, at the beginning of the incubation, is sufficient to perturb the formation of PrP 106–126 aggregates, resulting in shorter fibrils (mainly of 8 nm) that also accumulate in lower amounts (Fig. 1B). In equimolecular, or higher,

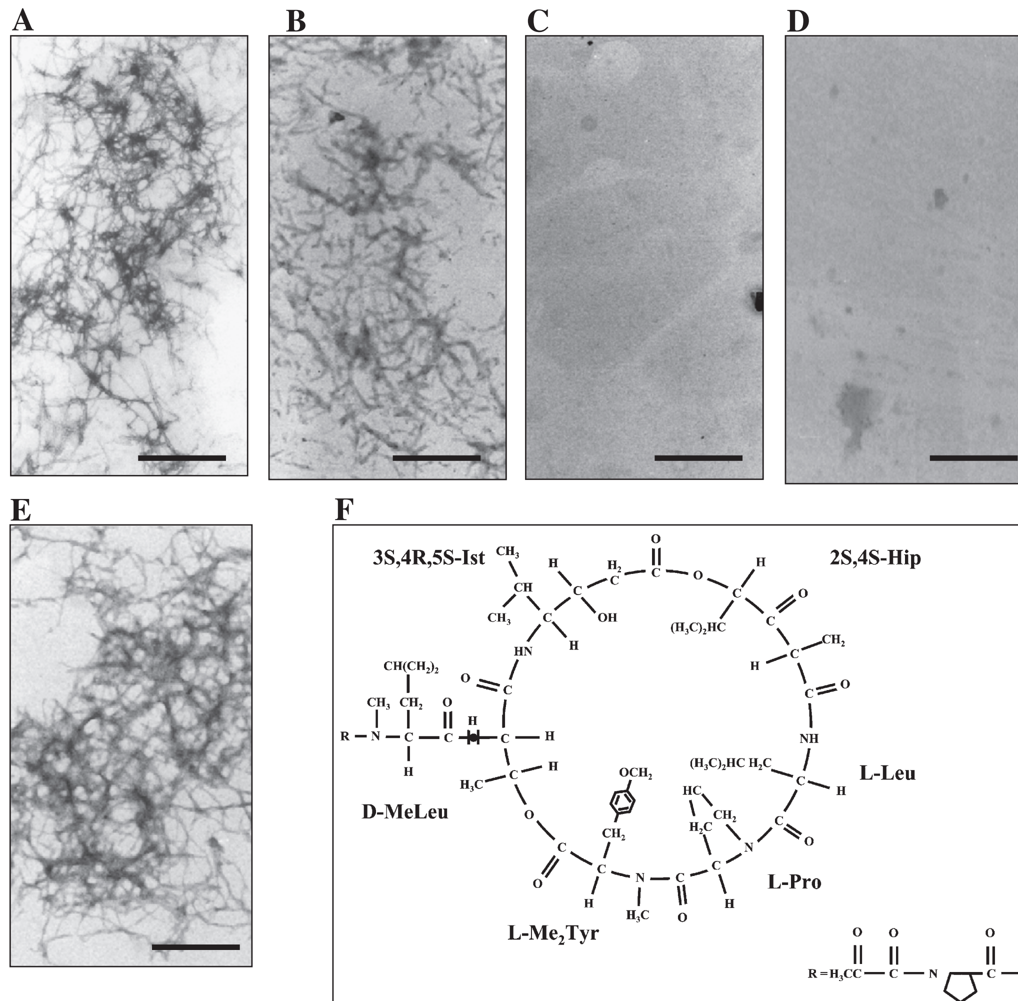

Fig. 1. Polymerization of PrP peptide in the absence and presence of Aplidine. All the in vitro polymerization experiments were carried out at final PrP 106–126 concentrations of ~ 430 μm (i.e. 1 mg/ml) as described in Ref. [15]. (A) PrP 106–126 aggregates incubated in the absence of Aplidine. (B) As in A, but co-incubated with Aplidine at a final concentration of 50 μM, (C) as in A, but co-incubated with Aplidine at a concentration of 500 μM. (D) Aplidine alone. (E) PrP 106–126 aggregates co-incubated with 10 mM proline. (F) Schematic representation of the chemical structure of Aplidine. Bars indicate 500 nm.

concentrations of PrP 106–126 and Aplidine, no significantly sized aggregates could be detected by electron microscopy (Fig. 1C). Quantification of the amount of aggregated material by measuring the amount of sedimented peptide after centrifugation [15] confirms these conclusions (data not shown). As a negative control, we performed the same assay with only Aplidine and no PrP peptide, which rendered no aggregates detectable by electron microscopy (Fig. 1D). Because Aplidine contains several proline residues (see Fig. 1F), it could be argued that the inhibitory effect of Aplidine on the aggregation of PrP 106–126 is simply produced by non-specific binding to PrP 106–126 through its β-breaking proline residues. In fact, a previous report shows that it is possible to dissolve PrP^{sc} by co-incubation with a ~ 1000 M excess of a peptide mimetic of the 115–122 sequence of PrP that incorporates several prolines [18]. However, the inhibitory effect of Aplidine is not due to proline-mediated interactions with the PrP sequence. This is indicated by the lack of observable inhibition of PrP 106–126 aggregation by co-incubation with 10 mM proline (Fig. 1E). Like in the proline-rich analogs of PrP 115–122 [18], proline residues in Aplidine are probably involved in breaking the network of hydrogen bonds required for growth of β-sheets, and not in providing binding energy. Aplidine at 500 μM concentration is also able to partially dissolve previously assembled PrP 106–126 fibrils (Fig. 2), suggesting that the formation of PrP fibrils is thermodynamically controlled.

To investigate the potential specificity of Aplidine as an inhibitor of PrP 106–126 aggregation, we carried out similar experiments with another peptide that aggregates into β-sheet containing fibrils. As it is shown in Fig. 3A, the 25–35 fragment of the β-amyloid peptide forms well defined long fibrillar aggregates when incubated in aqueous solution. Co-incubation with Aplidine results in changes in the apparent morphology of the aggregates under the electron microscope. The fibrillar aggregates of β-amyloid 25–35 become thinner and form bundles (Fig. 3B, C and

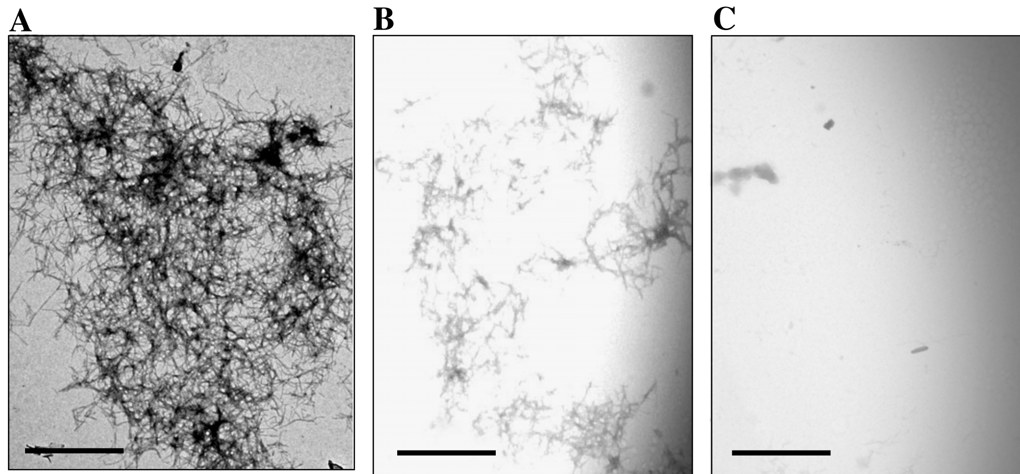

Fig. 2. Effect of Aplidine on PrP peptide polymers. PrP peptides assembled as indicated in Materials and methods, were incubated in the absence (A), for 1 h (B), and 2 h (C) at 37 °C. (in the presence of 500 μM Aplidine). Bars indicate 200 nM.

D). The changes are already observable at low Aplidine concentrations, and become more apparent as the concentration of Aplidine increases. However, fibrillar aggregates can be observed in all the conditions investigated.

One of the critical characteristics of the prion infectivity model is the existence of a conformational change into β-sheet structure. The conformational change is thought to trigger aggregation, leading to formation of infectious particles [3]. PrP 106–126 molecules in the fibrillar aggregates have a high content of β-sheet structure [14]. This is clearly observed in the amide II region of the FTIR spectrum of PrP 106–126 (Fig. 4A). The amide II band of PrP 106–126 shows a very intense maximum in the region near 1620 cm^{-1} and a weak maximum at ~ 1680 cm^{-1}, both indicative of a high content of hydrogen-bonded β-sheet structure. The FTIR spectrum also shows the pres-

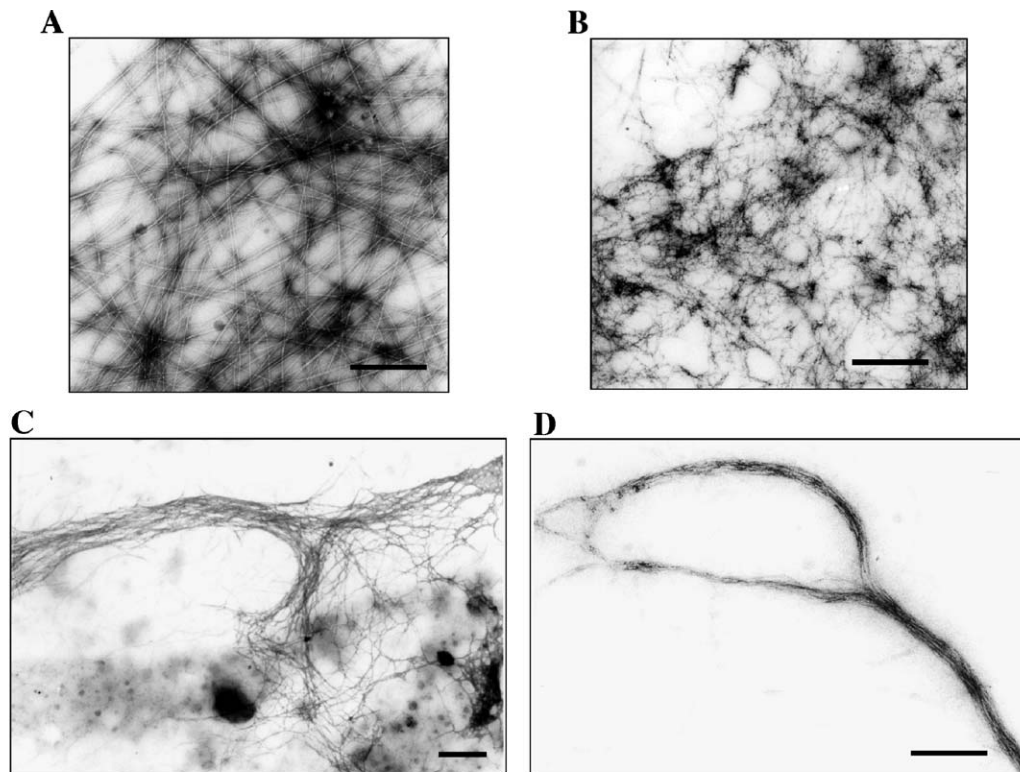

Fig. 3. Polymerization of beta amyloid peptide in the presence and absence of Aplidine. (A) Polymerization of β-amyloid peptide in aqueous solution as described in Materials and methods. (B) Polymerization of β-amyloid peptide in the same conditions, but in presence of 50 μM Aplidine, (C) 500 μM Aplidine, or (D) 1 mM Aplidine. Bars indicate 500 nm.

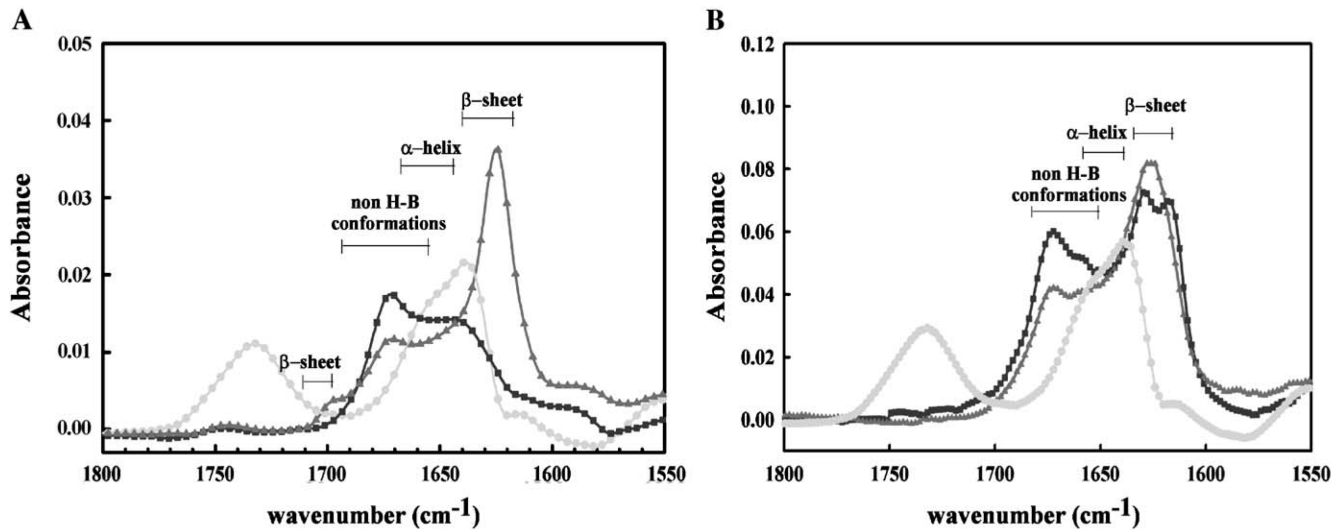

Fig. 4. FTIR of PrP 106–126 and β-amyloid peptides in the presence and absence of Aplidine. (A) Amide II region of the FTIR spectrum of peptide PrP 106–126 in aqueous solution in conditions leading to formation of β-sheet fibrils (triangles), of Aplidine alone (circles), and of peptide PrP 106–126 in the same conditions, but incubated with a 4-fold molar excess of Aplidine (squares). The last spectrum was obtained as the difference between the FTIR spectrum of the mixture PrP 106–126/Aplidine and that of Aplidine alone. Spectral regions typical of β-sheet, α-helix and non-hydrogen-bonded conformations are shown for comparison. (B) As in A, but for the β-amyloid peptide. Amide II region of the FTIR spectrum of β-amyloid in aqueous solution in conditions leading to the formation β-sheet fibrils (triangles), of Aplidine alone (circles), and of β-amyloid in the same conditions, but co-incubated with a 4-fold molar excess of Aplidine (squares).

ence of a minor amount of unstructured (i.e. non-hydrogen bonded) conformations. This can arise from some fraying of the ends of the PrP 106–126 peptide in the β-sheet aggregate, or from the presence of a small proportion of soluble monomeric PrP 106–126 peptide. Co-incubation with a 4-fold mass excess of Aplidine produces a drastic change in the amide II region of PrP 106–126. The two maxima mentioned above disappear, while the intensity of the area corresponding to non-hydrogen-bonded conformations increases significantly (Fig. 4A). All of this indicates that upon addition of Aplidine, PrP 106–126 shifts from β-sheet structure to a set of unstructured conformations. β-amyloid 25–35 also contains β-sheet structure in its fibrillar aggregated form. The structure of aggregated β-amyloid 25–35 is, however, more heterogeneous. FTIR reveals again a maximum at 1620 cm^{-1}, but with lower relative intensity than for PrP 106–126. The weak maximum at 1680 cm^{-1} is not observed, and the intermediate bands corresponding to non-hydrogen-bonded conformations are, in this case, more intense (Fig. 4B). Interestingly, co-incubation with Aplidine results in only slight changes in the conformation of β-amyloid 25–35. The decrease in the intensity at 1620 cm^{-1} and the increase of the intensity at $\sim$ 1665 cm^{-1} indicate a very small increase in the structural heterogeneity of β-amyloid 25–35. This is consistent with the electron microscopy images of the material, which show fibrillar aggregates in the presence of Aplidine (see above), and further supports the idea that the inhibition of the aggregation of PrP 106–126 induced by Aplidine is much stronger than that observed on β-amyloid peptide aggregation.

4. Discussion

Our results show that Aplidine inhibits the aggregation of PrP 106–126 and the formation of β-sheet structure that occurs concomitantly. The inhibitory effect is already detectable at concentrations of Aplidine of only 12% of that of PrP 106–126, while total inhibition only requires a 1:1 molar ratio. In principle, two mechanisms are conceivable to explain these results. In the first one, Aplidine inhibits PrP 106–126 aggregation by strongly binding to the monomeric peptide, sequestering it from the aggregation reaction. The second mechanism involves binding of Aplidine to the growing ends of the fibrillar aggregates, thereby blocking further growth. In the first mechanism, to obtain full inhibition of aggregation with a 1:1 ratio of Aplidine–PrP, the binding affinity of Aplidine for PrP would have to be much greater than that of PrP for itself. Furthermore, it is difficult to explain the observation of inhibitory effects at low concentrations of Aplidine with a simple monomer-sequestering mechanism. The second mechanism can naturally explain both observations, because a small amount of Aplidine will result in effective reduction of fibril formation by just transiently capping very short 'nucleated' fibrils, even without a strong affinity to bind PrP 106–126. This mechanism results in as stronger inhibitory effect and a potentially more powerful therapeutic agent. Moreover, the inhibition of PrP 106–126 aggregation by Aplidine is rather specific. Under the same conditions tested on PrP 106–126, Aplidine does not significantly inhibit the aggregation of the β-amyloid 25–35 peptide. Such preferential action on PrP 106–126 aggregates reveals that the inhibitory effect is

induced by selective binding to some amino acidic residues of the PrP 106–126 sequence rather than produced by a more generic β-breaker ability of Aplidine. Other inhibitors of prion aggregation that have been described previously rely on closely mimicking the original prion sequence to obtain selective binding [17,18]. Aplidine is in fact the first specific inhibitor that has no sequence homology with the prion sequence.

The finding of clinical correlations between the observation of β-sheet protein aggregates deposited in brain tissue and the onset of prion-related and many other diseases has resulted in a variety of efforts to identify inhibitors of these aggregation processes to be used as therapeutic agents. More recent ideas suggest that the agents causing these diseases are not the aggregates themselves, but perhaps small oligomeric precursors of the fibrillar aggregates [1]. Even if this is the case, the underlying mechanism of formation of the oligomeric precursors is presumably still the same, involving a conformational change into β-structure followed by intermolecular association. Interestingly, in a recent sophisticated kinetic study of amyloid formation, it has been found that aggregation occurs in two phases: first, by the formation of oligomers, then followed by their accretion into long fibrillar aggregates [24]. Moreover, the large aggregates could play a depository role by forming structures that withstand degradation by proteases from which oligomers are released slowly. Therefore, finding blockers of β-sheet aggregation is still a promising strategy to develop therapeutic agents for these diseases. In this regard, Aplidine is particularly promising as a leading compound for drug development efforts. Previously found inhibitors are based on the sequence of the prion molecules [18], raising the possibility of cross-reactions. An excess of ∼ 1000 of the blocking peptide was required to achieve effective inhibition [18], compared to the 1:1 ratio that we find for Aplidine. Moreover, all these molecules were standard L-amino acid based peptides. Such peptides are typically not good candidates for drug development because they have a low tendency to cross the plasmatic membrane and are very sensitive to the attack of extracellular proteases. Aplidine is an anti-tumoral agent used in proliferating cells [19], which by means of its cyclic structure, crosses the membrane rather effectively and is resistant to protease degradation. The direct use of this compound for therapeutic purposes should be further tested to determine whether its toxicity level is sufficiently low in neural cells. Alternatively, Aplidine can be used as a lead compound to develop derivatives that maintain its preferential inhibitory effect on PrP aggregation but reduce its potential toxicity.

In summary, Aplidine is a strong inhibitor of the assembly of the prion peptide 106–126 into β-sheet containing fibrils that works at equimolecular concentrations. Moreover, the strength of the inhibitory effect relies on specific interactions between Aplidine and the amino acidic sequence of PrP 106–126.

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