Molecular Mechanisms of Neurodegeneration

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'Nature-inspired' drug–protein complexes as inhibitors of Aβ aggregation

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Abstract

Protein–protein interactions are a regulatory mechanism for a number of physiological and pathological cellular processes. Neurodegenerative diseases, such as AD (Alzheimer's disease), are associated with the accelerated production or delayed clearance of protein aggregates. Hence, inhibition of pathologic protein–protein interactions is a very attractive mechanism for drug development. This review focuses on a novel therapeutic strategy to inhibit the *de novo* formation of protein aggregates. Inspired by strategies used in Nature and optimized over millions of years of evolution, we have created a bifunctional molecule [SLF (synthetic ligand for FK506-binding protein)–CR (Congo Red)] that is able to block Aβ (amyloid β) aggregation by borrowing the surface and steric bulk of a cellular chaperone.

Introduction

The increase in the average life expectancy of humans has focused attention on the rise in the prevalence of age-related neurodegenerative diseases. AD (Alzheimer's disease) is the most common of these diseases, and the sporadic form of AD affects approx. 5–10% of the population above the age of 65 years. AD patients suffer from dementia characterized by memory loss, inability to perform daily activities, language impairment and behavioural abnormalities. Approx. 10% of all AD cases are early-onset FAD (familial AD) and have already developed severe dementia by midlife. Over the next few decades, AD is predicted to become a major public health problem [1].

Key words: aggregation, amyloid β, chaperone, FK506-binding protein, linker, small-molecule ligand.

Abbreviations used: Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; CR, Congo Red; FAD, familial AD; FKBP, FK506-binding protein; mTOR, mammalian target of rapamycin; SLF, synthetic ligand for FK506-binding protein; TCR, T-cell receptor.

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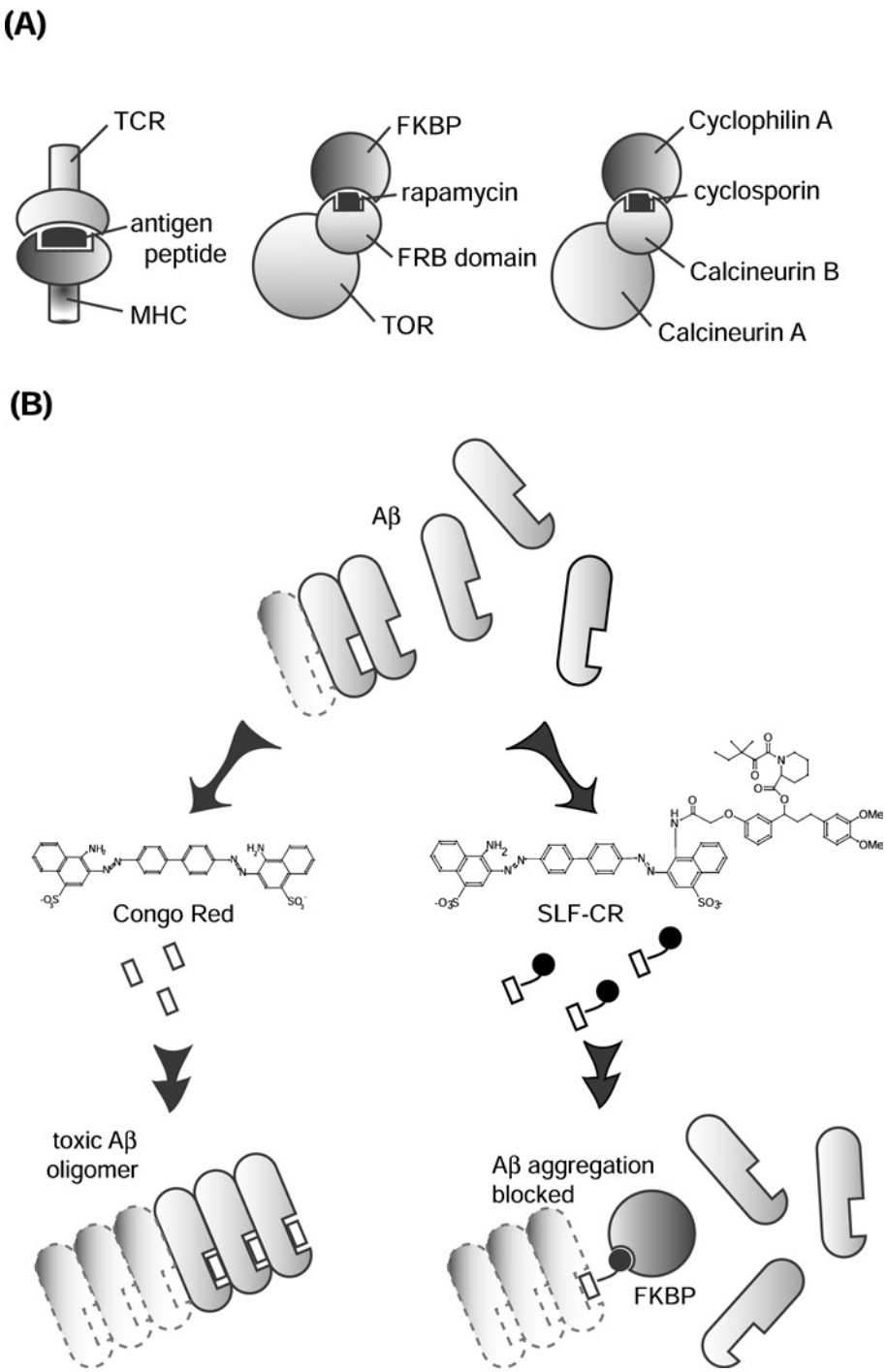
AD causes neuronal degeneration primarily in the hippocampus and basal forebrain. The hallmarks of AD are senile plaques of Aβ (amyloid β) peptide and neurofibrillary tangles composed of hyperphosphorylated protein tau [2,3]. The Aβ peptide itself is produced via the sequential proteolytic processing of APP (amyloid precursor protein) by first β and then γ secretases [4]. APP is a ubiquitously expressed transmembrane glycoprotein whose physiological function is unclear. Genetic studies have linked early-onset FAD to mutations in three genes, APP, PS1 (presenilin 1) and PS2 (presenilin 2) [5]. These mutations increase either the absolute level of Aβ or the relative amount of the longer, more amyloidogenic form of Aβ (Aβ 1–42). Genetic, molecular, biochemical and transgenic studies over the last two decades gave rise to the amyloid hypothesis, which suggests that the accumulation of Aβ is an initial pathogenetic factor for the development of neurodegeneration in AD [6,7]. Owing to the poor correlation between neuritic plaques and cognitive deficits [8], it remains uncertain to what extent extracellular amyloid deposits contribute to loss of neuronal viability or function in AD. Thus, recently, increased attention has

Figure 1 | A novel anti-Aβ aggregation strategy

(**A**) Examples of ternary complex formation in biology. FRB, FKBP rapamycin-binding domain. (**B**) Model of inhibition of Aβ aggregation using a bifunctional molecule. A bifunctional molecule recruits a protein, FKBP, which increases the drug's effective molecular mass. The newly acquired steric bulk of the drug enables it to inhibit the progress toward Aβ aggregation. In comparison a small molecule (minus the recruitment arm) fails to slow Aβ aggregation.

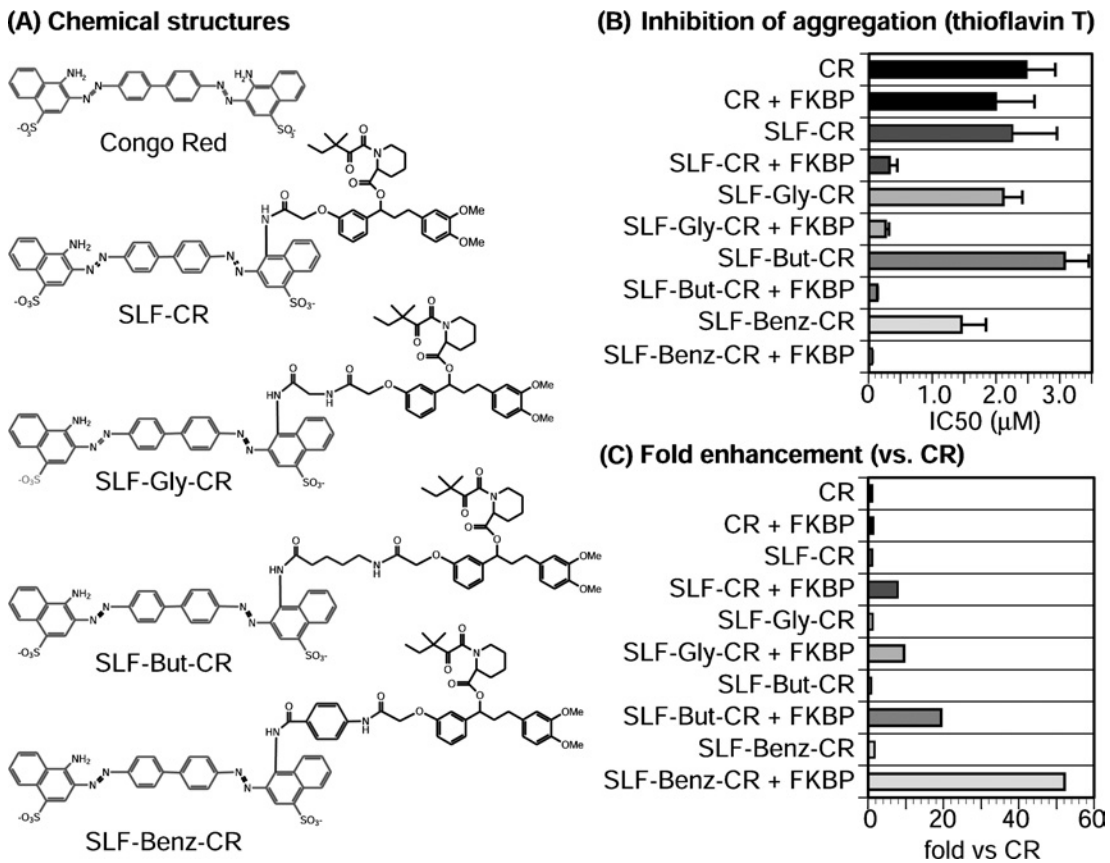


focused on the role of immature, oligomeric forms of the Aβ in the pathogenesis of AD.

Aβ peptides are prone to self-aggregation, *in vivo* and *in vitro* [9,10]. Mature amyloid fibrils are filamentous with a cross β-sheet structure. The β-strand is perpendicular to the axis of the filament, so that the hydrogen bonding is parallel to the axis. As Aβ aggregates impair neuronal viability and function [11–13], drugs that inhibit the aggregation process

Figure 2 | Studies towards inhibition of Aβ oligomerization using bifunctional molecules

(**A**) Chemical structures of the bifunctional molecules used in our study. The Aβ target (CR) is shown in grey, the recruitment domain (SLF) is in black and the variable linker region is shown between. (**B**) Inhibition of Aβ fibrillogenesis as measured by thioflavin T fluorescence. The average IC_{50} for two experiments performed in triplicate is shown for the bifunctional inhibitors. FKBP was added to each aggregation reaction at a final concentration of 1 μM. (**C**) Relative potency of the bifunctional linker series depicted as fold enhancement (compared with that of CR).



may serve as effective therapeutics. Unfortunately, protein–protein interactions, especially those between amyloidogenic proteins, have proven exceedingly difficult to inhibit.

However, combination therapies that target multiple steps in the pathogenesis of AD may be required for effective treatment; therefore pursuit of aggregation inhibitors remains an important goal [14–18]. There are several reasons why disrupting protein–protein interaction with small molecules has always been a serious hurdle for the pharmaceutical industry. The binding energy that drives protein–protein contacts is distributed over the extended face of the protein, and an array of electrostatic, hydrophobic and hydrogen bonding interactions contribute towards it. In addition, protein interfaces are often transient and dynamic. Only a fraction of them have well-defined hot spots for drug binding. We hypothesize that, owing to the complexity of forces that drive protein–protein interactions, it is possible that conventional small molecules have insufficient steric bulk.

A novel anti-Aβ aggregation strategy: harnessing chaperones

Nature uses a remarkable strategy that allows small-molecule ligands to enhance the affinity for their target receptors. They often bind to an endogenous protein first and then present a composite surface to the target protein for interaction. For example, peptide ligands for the TCR (T-cell receptor) are presented by the MHC to the TCR. Alone, the peptides exhibit a low affinity for the TCR. Both structural [19] and mutation [20] studies have shown convincingly that these TCR–MHC contacts are critical for the remarkable specificity of the immune response. Some micro-organisms have evolved an extraordinary mechanism to enhance the affinity and efficacy of small-molecule ligands. For example, the immunosuppressive drugs cyclosporin and FK506 bind to the peptidyl-prolyl *cis–trans* isomerases cyclophilin and FKBP (FK506-binding protein) respectively to form a new composite surface and thus inhibit the activity of their target,

calcineurin. By themselves, FK506 and cyclosporin have no measurable affinity for calcineurin. Yet, the protein–drug complexes bind to calcineurin with high affinity and are nanomolar inhibitors of its activity. Similarly, the macrolide rapamycin has no measurable affinity for its target mTOR (mammalian target of rapamycin), a protein important for cell growth. Instead, rapamycin first binds to FKBP, resulting in a drug–protein complex which then binds to mTOR with high affinity [21,22].

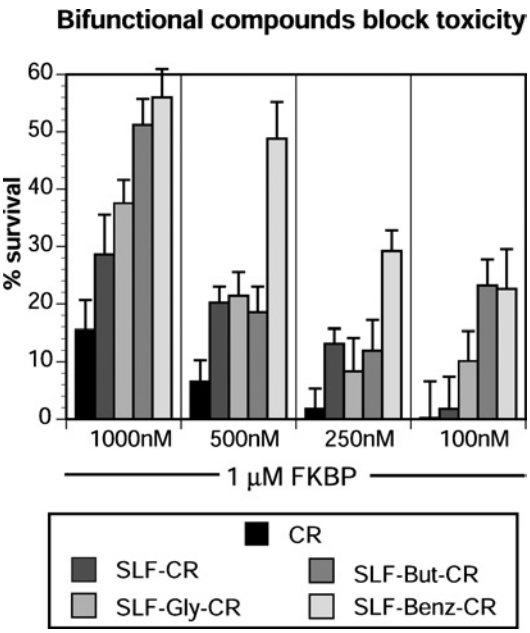
Inspired by Nature, we envisioned chemically derivatizing an Aβ-binding small molecule with a known high-affinity ligand of an endogenous protein [23,24]. The resultant bifunctional molecule has an amyloid-binding moiety as well as a recruitment moiety for a cellular chaperone. We hypothesized that recruitment of steric bulk of the endogenous protein will empower the small bifunctional molecule to interfere with protein–protein contacts of the oligomerizing Aβ (Figure 1).

To explore the feasibility of our approach, we chose FKBP as the presenter protein. The FKBPs are a ubiquitous class of peptidyl-prolyl *cis–trans* isomerases encoded by 23 different genes which are all highly expressed in mammalian cells [25]. They have a wide tissue distribution and low-nanomolar ligands for this family of chaperones are available. For our strategy, chaperones are ideal candidates as presenter proteins. Their surfaces have evolved to bind many proteins, especially exposed hydrophobic surfaces of folding and misfolded intermediates. Thus we chose the synthetic ligand SLF (synthetic ligand for FKBP) for recruitment of FKBP and the dye CR (Congo Red) as the amyloid ligand. CR has been used as an amyloid-specific stain and inhibitor of aggregation for many years [26]. Alone, CR is an unsuitable drug candidate owing to the high concentration necessary to block Aβ fibrillogenesis. We hypothesized that this modest potency arises from the insufficient bulk of CR to efficiently block the large contact surface of the Aβ–Aβ interaction. We expected that the bifunctional molecule SLF–CR would recruit FKBP and that the drug–protein complex would gain both in affinity for Aβ and in potency as an inhibitor. Moreover, we expected to improve the efficacy of the inhibitors by varying the length and flexibility of the linker between CR and SLF. To test these hypotheses, we synthesized the bifunctional ligands as shown in Figure 2(A).

We explored the inhibition of Aβ oligomerization by the well-known thioflavin T assay [27]. The fluorescence of thioflavin T at 490 nm is enhanced dramatically in the presence of Aβ aggregates. We found that the IC_{50} of the SLF–CR + FKBP combination was approx. 5-fold lower than that of CR + FKBP for reducing the concentration of thioflavin-T-binding species (Figure 2B). Additionally, the assay revealed that the nature of the linker significantly influenced the potency of the bifunctional molecule. These studies showed that SLF–Benz-CR + FKBP was the most active compound, with an IC_{50} of approx. 50 nM. It was remarkable that this simple modification of just changing the linker was sufficient to generate a compound with an IC_{50} that is 40-fold better than the monomeric compound CR + FKBP and a 6-fold improvement over the parent bifunctional molecule, SLF–CR + FKBP (Figure 2C). These results were confirmed by other biophysical studies such as light scattering, AFM (atomic force microscopy) and TEM (transmission electron microscopy).

Figure 3 | Cell viability of primary rat hippocampal neurons as measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay

Dose-dependent detoxification of Aβ by bifunctional compound series.



From a therapeutic standpoint, we decided to evaluate the potential of our bifunctional drugs to prevent the formation of neurotoxic Aβ aggregates. So we examined whether the bifunctional drugs could inhibit neurotoxicity of *in vitro* aggregated Aβ on primary neurons. Cell viability of P0 rat hippocampal neurons was assessed with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] reduction assay [28]. SLF–CR + FKBP-treated Aβ samples were substantially less toxic than untreated or CR + FKBP-treated samples. Specifically, SLF–CR + FKBP-treated Aβ samples displayed an EC_{50} approx. 4-fold better than CR/FKBP (approx. 0.9 and 4.2 μM respectively). Furthermore, the ability of SLF–CR to prevent neuronal death was dependent on the concentration of FKBP. This strongly supports that drug-mediated recruitment of FKBP also inhibits neuronal toxicity. Consistent with our observations in the thioflavin T assay, SLF–Benz-CR + FKBP and SLF–But-CR + FKBP exhibited significantly higher potency than CR + FKBP or SLF–CR + FKBP (Figure 3).

Conclusion

Inspired by natural examples of protein–ligand–protein interactions, we have developed a novel strategy wherein the recruitment of a presenter protein by a bifunctional molecule

significantly blocks Aβ aggregation. The design of the bifunctional drug is modular and features: (i) one half that allows recruitment of the chaperone/presenter protein; (ii) a linker whose length and rigidity may be varied; and (iii) a remaining half that constitutes a ligand which binds to Aβ. Changes to any of these three variables can significantly modulate the potency and specificity of our bifunctional anti-Aβ aggregation inhibitors. Ultimately, we envision that such a strategy is potentially extendable to develop small-molecule agonists or antagonists of many protein–protein interactions.

References

1 World Health Organization (2002) Active ageing: a policy framework, Second United Nations World Assembly on Aging, Madrid, Spain (World Health Organization, Geneva, 2002)
2 Glenner, G.G. and Wong, C.W. (1984) Biochem. Biophys. Res. Commun. **120**, 885–890
3 Lee, V.M.-Y., Balin, B.J., Otvos, Jr, L. and Trojanowski, J.Q. (1991) Science **251**, 675–678
4 Haass, C., Schlossmacher, M., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B. et al. (1992) Nature (London) **359**, 322–325
5 Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W. et al. (1996) Nat. Med. **2**, 864–870
6 Hardy, J. and Selkoe, D.J. (2002) Science **297**, 353–356
7 Koo, E.H., Lansbury, P.T. and Kelly, J.W. (1999) Proc. Natl. Acad. Sci. U.S.A. **96**, 9989–9990
8 Braak, H. and Braak, E. (1998) J. Neural Transm. Suppl. **53**, 127–140
9 Murphy, R.M. (2002) Annu. Rev. Biomed. Eng. **4**, 155–174
10 Caughey, B.M. and Lansbury, P.T. (2003) Annu. Rev. Neurosci. **26**, 267–298
11 Mucke, L., Masliah, E., Yu, G.-Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K. and McConlogue, L. (2000) J. Neurosci. **20**, 4050–4058
12 Kim, H.J., Chae, S.-C., Lee, D.-K., Chromy, B., Lee, S.C., Park, Y.C., Klein, W.L., Krafft, G.A. and Hong, S.T. (2003) FASEB J. **17**, 118–120
13 Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L. et al. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 6448–6453
14 Wong, G.T., Manfra, D., Poulet, F.M., Zhang, Q., Josien, H., Bara, T., Engstrom, L., Pinzon-Ortiz, M., Fine, J.S., Lee, H.J. et al. (2004) J. Biol. Chem. **279**, 12876–12882
15 Citron, M. (2004) Trends Pharmacol. Sci. **25**, 59–112
16 Orgogozo, J.M., Gilman, S., Dartigues, J.F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S. et al. (2003) Neurology **61**, 46–54
17 McGeer, P.L., Schulzer, M. and McGeer, E.G. (1996) Neurology **47**, 425–432
18 Jick, H., Zornberg, G.L., Jick, S.S., Seshadri, S. and Drachman, D.A. (2000) Lancet **356**, 1627–1631
19 Garcia, K.C., Degano, M., Stanfield, R.L., Brunmark, A., Jackson, M.R., Peterson, P.A., Teyton, L. and Wilson, I.A. (1996) Science **274**, 209–219
20 Ajitkumar, P., Geier, S.S., Kesari, K.V., Borriello, F., Nakagawa, M., Bluestone, J.A., Saper, M.A., Wiley, D.C. and Nathenson, S.G. (1988) Cell **54**, 47–56
21 Choi, J., Chen, J., Schreiber, S.L. and Clardy, J. (1996) Science **273**, 239–242
22 Liu, J., Farmer, Jr, J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Cell **66**, 807–815
23 Gestwicki, J.E., Crabtree, G.R. and Graef, I.A. (2004) Science **306**, 865–869
24 Kelly, J.W. (2005) N. Eng. J. Med. **352**, 722–723
25 Galat, A. (2003) Curr. Topics Med. Chem. **3**, 1315–1347
26 Westermark, G.T., Johnson, K.H. and Westermark, P. (1999) Methods Enzymol. **309**, 3–25
27 LeVine, III, H. (1999) Methods Enzymol. **309**, 274–284
28 Shearman, M.S. (1999) Methods Enzymol. **309**, 716–723

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