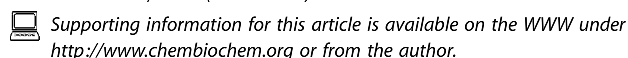
Inhibition of Fibril Formation of Aβ by Guanidiniocarbonyl Pyrrole Receptors

Carsten Schmuck,*[a] Peter Frey,[b] and Martin Heil[a]

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. Pathology visible "post mortem" includes neurodegeneration and extracellular deposition of amyloids, both in neuritic plaques and diffuse deposits.[1] The major proteinaceous component of AD amyloids is the amyloid-β protein (Aβ), a protein consisting of 39–42 amino acids derived from the Alzheimer precursor protein (APP).[2] Inhibition of the formation of β-amyloid fibrils, formed by self assembly from this amyloid β-peptide is an attractive target for the treatment of Alzheimer's disease.[3] In the last few years, various small molecules such as rifampicin, Congo red, curcumin, and apomorphin have been shown to inhibit Aβ aggregation both in vitro and in cell assays.[4] Besides these molecules, which are rather unspecific and whose mode of action is completely unclear, only very few examples of designed β-sheet breakers are known so far.[5] These are mainly oligopeptides, representing fragments of Aβ itself. For example, Kiessling used Tjernbergs'[5g] oligopeptide KLVFF, which is based on one of the self-assembling recognition sequences of the amyloid peptide (residues 16–20) and added positively charged lysines to enhance the solubility of the aggregates.[5e,j] Soto's pentapeptide amides were also based on the KLVFF recognition sequence of Aβ.[5h,i] Hetényi showed that the cationic pentapeptide amide RVVIA, which is based on the C-terminal sequence VVIA of Aβ (1–42), also interferes with fibril formation.[5c,d,f] All these oligopeptides require millimolar concentrations and an up to 20-fold excess relative to Aβ (1–42) to reduce the amount of fibrils by 40% at best. Unfortunately, very little is known about their mechanism of action, their exact binding specifities or the mo-

[a] Prof. Dr. C. Schmuck, Dr. M. Heil
Institut für Organische Chemie, Universität Würzburg
Am Hubland, 97074 Würzburg (Germany)
Fax: (+49) 931-888-4625
E-mail: schmuck@chemie.uni-wuerzburg.de

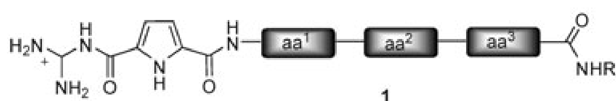
[b] Dr. P. Frey
Novartis AG, Basel (Switzerland)


Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/cbic.200400270 ChemBioChem 2005, 6, 628–631

lecular basis of their interaction with Aβ, which is a major obstacle in the design of more-specific and efficient amyloid inhibitors for potential future therapeutic use. It is not even clear whether the exact binding sites of these oligopeptides are indeed the complementary recognition sequences of Aβ (1–42) for which they were initially designed.

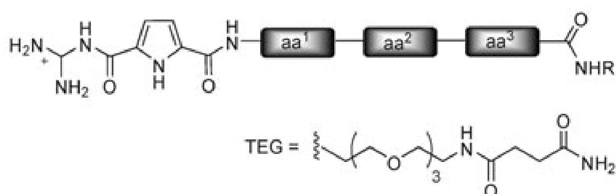
We have recently introduced a new class of artificial receptors **1** capable of binding the anionic model tetrapeptide VVIA,



representing the free C terminus of the amyloid β-protein Aβ (1–42).[6] These receptors consist of a guanidiniocarbonyl pyrrole cation, a highly efficient binding motif for carboxylates even in aqueous solvents,[7] and a variable tripeptide unit.

By using a combinatorial receptor library and a UV binding assay, efficient receptors for the binding of VVIA under various conditions were identified. The best receptors showed binding constants up to $K=5\times10^3\,\text{M}^{-1}$ for this model peptide.[6] As the hydrophobic C terminus of Aβ (1–42) is critical for fibril formation, we thought that our selective receptors for this VVIA sequence could influence the capability of fibril formation of Aβ (1–42). Herein, we therefore wish to report that receptors of type **1** indeed inhibit fibril formation of Aβ (1–42) in vitro by selectively binding to its C terminus, as could be shown by studying their effect on the fibrillogenesis of Aβ (1–42) in comparison to Aβ (1–40).

Six different tripeptide based guanidiniocarbonyl pyrrole receptors **2**–**7** (Scheme 1) were synthesized on Rink amide resin by using standard Fmoc protocol (see Supporting Information).



receptor	aa1	aa2	aa3	R
2	Val	Val	Val	H
3	Val	Val	Val	TEG
4	Lys	Leu	Lys	H
5	Lys	Tyr	Lys	H
6	Lys	Lys	Phe	H
7	Phe	Glu	Lys	H

Scheme 1. *Tripeptide based guanidiniocarbonyl pyrrole receptors tested for the inhibition of fibril formation of Aβ (1–42) and Aβ (1–40).*

We tested their ability to inhibit fibril formation in vitro with both Aβ (1–42) and Aβ (1–40) by two standard assays[8] using Thioflavin T[9] and Congo red,[10] as described by LeVine and Klunk. Thioflavin T interacts with aggregated Aβ and shows a bathochromic shift in the fluorescence spectrum. Upon interaction with the fibrils, a characteristic new fluorescence maximum at 482 nm occurs, as opposed to the emission at 445 nm of the free dye. This emission change occurs within seconds and is directly dependent on the fibril concentration. Therefore, the kinetics of fibril formation can be followed by simply measuring the time dependence of the increase in fluorescence of Thioflavin T.

Thereafter, the amount of fibrils was quantified by using the change of the UV absorption of Congo red upon binding to these fibrils. When Congo red binds to fibrillar Aβ, a change in color from orange-red to rose is induced, which corresponds to a shift of the absorption maximum of the dye from 480 to 541 nm. This shift depends on the aggregation state of the peptide. By comparing the absorption properties of both Congo red and the amyloid fibrils on the one hand and their mixture on the other at two different wavelengths, an absolute quantification of the amount of fibrils formed is possible according to the method derived by Klunk.[10]

The six receptors **2**–**7** were chosen based on our previous binding studies with the model tetrapeptide VVIA.[6] The trivaline receptor **2** was identified as the best binding receptor among a combinatorial library of 512 members from a screening in methanol.[5a] The tri(ethylene glycol) (TEG) unit in **3** was attached to increase solubility. Receptor **4** was identified as the best binding sequence from a screening in water.[6b] Receptors **5** and **6** were not members of our initial library but were chosen as structural analogues of **4** as they also contain two positively charged lysines. In receptor **7**, one of these two lysines was exchanged for a negatively charged glutamate, thus keeping the receptor highly polar but reducing it net charge relative to **4**–**6**.

Indeed, at 1 mM, the receptors **5** and **6** showed an inhibitory effect in the Thioflavin T assay for Aβ (1–42) with a slightly prolonged lag period of approximately four hours. Fluorescence values reached a maximum after 1 day and remained at a plateau thereafter. Both receptors also significantly reduced the total fluorescence values. A typical set of data is shown in Figure 1 for receptor **5**. Receptors **2**, **4**, and **7** did not show any effect in the Thioflavin T assay.

At the end of the assay, after six days, the amounts of Aβ (1–42) fibrils were quantified by Congo red binding. The data are summarized in Table 1 and in Figure 2. In agreement with

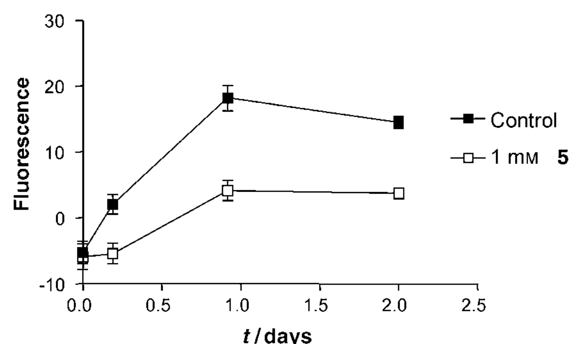


Figure 1. *Thioflavin T assay of **5** for Aβ (1–42).*

© 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

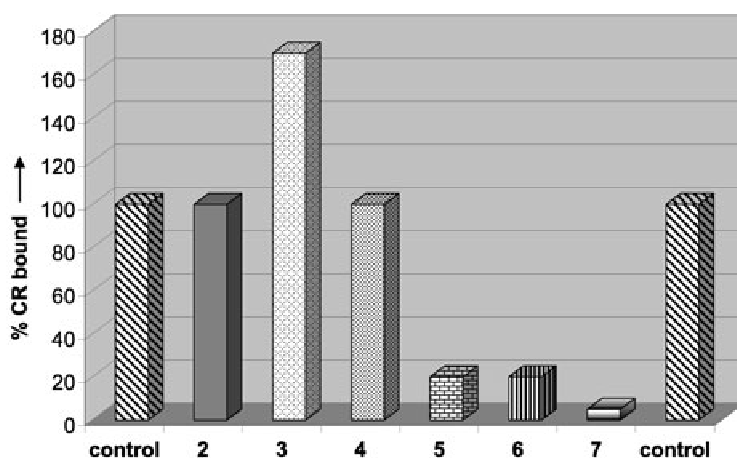


*Figure 2. Results of the Congo red (CR) assay for receptors **2**–**7** (1 mM) with Aβ (1–42).*

*Table 1. Results of the Congo red assay for receptors **2**–**7** with Aβ (1–42) and Aβ (1–40).*

	Receptor	Change in amount of fibrils	
		Aβ (1–42)	Aβ (1–40)
2	1 mM	no effect	no effect
	100 μM	no effect	no effect
3	1 mM	+170%	+340%
	100 μM	no effect	+50%
4	1 mM	no effect	no effect
	100 μM	no effect	no effect
5	1 mM	−80%	no effect
	100 μM	−95%	no effect
6	1 mM	−80%	no effect
	100 μM	−40%	no effect
7	1 mM	−95%	−95%
	100 μM	−35%	−39%

the Thioflavin T assay, receptors **2** and **4** did not have any effect on the amount of fibrils formed. Receptors **5** and **6**, which both retarded fibril formation in the Thioflavin T assay, also significantly reduced the amount of fibrils according to the Congo red binding. For example, in the presence of either 1 mM **5** or **6**, the amount of Congo red-stained fibrils was reduced by ≈−80%! Even in the presence of only 100 μM receptors (corresponding to only a threefold excess relative to Aβ), a significant reduction in the amount of fibrils was still observed (e.g. −40% for **6**). Receptor **7**, although it did not show any effect in the Thioflavin T assay, seems to reduce the amount of fibrils formed according to the Congo red binding assay. This apparently inconsistent behavior of **7** in the two assays shows that **7** probably does not interfere with fibril formation but interacts with Congo red thereby interfering with this assay (vide infra). Klunk already mentioned the possibility that a test compound could compete with Congo red for binding to Aβ. This would give the false appearance of inhibition of aggregation.

Surprisingly, the effect of the tri(ethylene glycol)-substituted receptor **3** on fibril formation completely differed from all the other receptors. According to the Thioflavin T assay **3** *accelerated* the fibril formation of Aβ (1–42) and *increased* the amount of amyloid plaques produced as determined by the Congo red assay (+170%).

These data show that artificial receptors such as **5** and **6** are indeed capable of interfering with fibril formation of Aβ (1–42) in vitro. To further probe the molecular basis of this effect, we also examined the ability of receptors **2**–**7** to inhibit fibril formation of Aβ (1–40), which lacks the C-terminal VVIA sequence, for which our receptors were designed. As the tendency of Aβ (1–40) to self-aggregate is less pronounced,[11] the critical concentration needed to form fibrils is higher than for Aβ (1–42), thus requiring a larger concentration of 100 μM peptide in the assay (compared to 33 μM for Aβ (1–42)). Therefore, the two receptor concentrations used correspond to a 1:1 and a 10:1 molar ratio for Aβ (1–40), instead of 3:1 and 30:1 for Aβ (1–42), but are still in the same molar range for both peptides.

Neither receptor **2** nor receptors **4**–**6** showed any significant effect on fibril formation of Aβ (1–40) either in the Thioflavin T or in the Congo red assay at either concentration tested. For receptor **7**, in a similar way to the situation with Aβ (1–42), again no effect was seen in the Thioflavin T assay, whereas the Congo red binding assay showed exactly the same effect of **7** on Aβ (1–40) as it did on Aβ (1–42). As before, the tri(ethylene glycol)-substituted receptor **3** accelerated the formation of fibrils also for Aβ (1–40) in the Thioflavin T assay and increased the total amount of fibrils by 340% at 0.1 mM concentration, as determined by the Congo red assay.

A comparison of the data for Aβ (1–42) and Aβ (1–40) suggests that the C terminus of Aβ (1–42) is indeed critical for the inhibition of fibril formation at least for receptors **5** and **6**. These two receptors do not have any effect on the formation of fibrils from Aβ (1–40) not even at a tenfold molar excess, but significantly inhibit fibrillogenesis of Aβ (1–42) both in the Thioflavin T and Congo red assay, even at a molar ratio as low as 3:1 relative to Aβ (1–42). A reasonable explanation is that both receptors **5** and **6** preferentially interact with the C-terminal VVIA sequence of Aβ (1–42), as expected from the model studies. Therefore, no effect is expected for Aβ (1–40), which lacks this sequence. Due to the polar character of the receptors, the solubility of the resulting supramolecular aggregates upon binding to the C terminus of Aβ (1–42) is probably increased sufficiently to inhibit fibril formation significantly.

That neither receptor **2** nor **4**, which both strongly interact with the C-terminal model tetrapeptide VVIA in solution,[6] affect fibril formation is surprising at first glance. However, this only underlines the fact that binding to the amyloid peptide is necessary but not sufficient to inhibit fibrillogenesis. The inhibition of fibril formation requires, at least, both binding to the peptide on the one hand and solubilizing properties on the

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

other. It is reasonable to assume that both receptors **2** and **4** interact with Aβ (1–42)—probably even selectively with its C terminus—but they are evidently not capable of increasing the solubility of the fibrils. In the case of **2**, this is not surprising, in view of its highly hydrophobic character. Why even **4**, despite the two charged lysines, is ineffective while the structurally related **5** and **6** are effective is not yet clear. But this points to the fact, that charge by itself does not guarantuee solubilization of the formed fibrils. Kiessling even observed that, for her hybrid peptides, fibril formation was enhanced with increasing number of lysines: the more polar the hybrid peptide was, the more fibrils were formed.[5e]

This effect is similar to the unexpected behavior of receptor **3**, which *favors* fibril formation in both assays. As the comparison with the ineffective receptor **2**, which has the same structure but lacks the tri(ethylene glycol) chain, indicates this must be obviously due to the tri(ethylene glycol) chain itself. Somehow this tri(ethylene glycol) unit both accelerates fibril formation (Thioflavin T) and also increases the amount of fibrils formed (Congo red) for both Aβ (1–42) and Aβ (1–40). This is in good agreement with earlier observations by Suhr who reported that poly(ethylene glycol) can actually induce amyloid formation in mice.[12] Therefore, much more has to be learned on a molecular basis to determine those factors that are crucial for the inhibition of fibril formation.

Receptor **7** shows the same inconsistent features in both assays with either Aβ (1–42) or Aβ (1–40): no effect is seen in the Thioflavin T assay, while **7** causes the same effect *even quantitatively* upon Congo red binding to both kinds of amyloid aggregate. This suggests that **7** either binds to another part of the amyloid peptide than the C terminus or somehow interferes with the binding of Congo red to the amyloid fibrils. If **7** bound to another part of Aβ than the C terminus, then again the same effect on fibril formation for Aβ (1–42) and Aβ (1–40) should be observed in the Thioflavin T assay. As this is not the case, rather, **7** shows no effect at all in the Thioflavin T assay, it is more likely that **7** interferes with the binding of Congo red to the fibrils. Hence, the results of the Congo red assay for **7** most likely do not indicate an inhibition of fibril formation, but represent a false positive. This underlines the use of a second independent assay as a control. Only if consistent behavior in both assays is observed (as is the case for all the other receptors studied here) can one be sure that the observed effects are probably due to inhibition of fibril formation and do not result from problems with the assay itself.[8]

In conclusion, we have demonstrated for the first time that specifically designed artificial receptors are capable of inhibiting amyloid fibril formation of Aβ (1–42) in vitro by interaction with its C-terminal VVIA sequence. Our results also show that polarity by itself is not enough to turn an efficient receptor into a potent β-sheet breaker. Much more has to be learned about which additional factors turn a good binder into an efficient inhibitor. But such work not only improves our understanding of peptide molecular interactions in general, but also opens an economic and easy way of identifying biologically active organic receptors by evaluating bioorganic model systems.

Acknowledgements

This work was supported by the Deutschen Forschungsgemeinschaft (DFG SCHM 1501/2-2) and the Fonds der Chemischen Industrie.

Keywords: artficial receptors · beta-sheet breakers · fibril formation · molecular recognition · peptides

[1] a) J. Hardy, D. J. Selkoe, *Science* **2002**, *297*, 353–356; b) L. Dumery, F. Bourdel, Y. Soussan, A. Fialkowsky, S. Viale, P. Nicolas, M. Reboud-Ravaux, *Pathol. Biol.* **2001**, *49*, 72–85; c) D. J. Selkoe, *Physiol. Rev.* **2001**, *81*, 741–766; d) R. Baumeister, S. Eimer, *Angew. Chem.* **1998**, *110*, 3148–3152; *Angew. Chem. Int. Ed.* **1998**, *37*, 2978–2982; e) D. J. Selkoe, *Science* **1997**, *275*, 630–631; f) J. Peter, T. Lansbury, *Acc. Chem. Res.* **1996**, *29*, 317–321.

[2] A. B. Clippingdale, J. D. Wade, C. J. Barrow, *J. Pept. Sci.* **2001**, *7*, 227–249.

[3] a) D. I. Dominguez, B. De Strooper, *Trends Pharmacol. Sci.* **2002**, *23*, 324–330; b) C. Adessi, C. Soto, *Drug Dev. Res.* **2002**, *56*, 184–193; c) J. P. T. Lansbury, *Curr. Opin. Chem. Biol.* **1997**, *1*, 260–267.

[4] a) K. Ono, K. Hasegawa, H. Naiki, M. Yamada, *J. Neurosci. Res.* **2004**, *75*, 742–750; b) H. A. Lashuel, D. M. Hartley, D. Balakhaneh, A. Aggarwal, S. Teichberg, D. J. E. Callaway, *J. Biol. Chem.* **2002**, *277*, 42881–42890; c) C. Soto, M. S. Kindy, M. Baumann, B. Frangione, *Biochem. Biophys. Res. Commun.* **1996**, *226*, 672–680.

[5] a) Y. Kuroda, K. Maeda, H. Hanaoka, K. Miyamoto, T. Nakagawa, *J. Pept. Sci.* **2004**, *10*, 8–17; b) P. Rzepecki, H. Gallmeier, N. Geib, K. Cernovska, B. König, T. Schrader, *J. Org. Chem.* **2004**, *69*, 5168–5178; c) C. Hetényi, Z. Szabo, E. Klement, Z. Datki, T. Körtvélyesi, M. Zarandi, B. Penke, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 931–936; d) C. Hetényi, T. Körtvélyesi, B. Penke, *Bioorg. Med. Chem.* **2002**, *10*, 1587–1593; e) T. L. Lowe, A. Strzelec, L. L. Kiessling, R. M. Murphy, *Biochemistry* **2001**, *40*, 7882–7889; f) C. Hetényi, T. Körtvélyesi, B. Penke, *J. Mol. Struct.* **2001**, *542*, 25–31; g) L. O. Tjernberg, D. J. E. Callaway, A. Tjernberg, S. Hahne, C. Lilliehöök, L. Terenius, J. Thyberg, C. Nordstedt, *J. Biol. Chem.* **1999**, *274*, 12619–12625; h) C. Soto, *Mol. Med. Today* **1999**, *5*, 343–350; i) C. Soto, M. S. Kindy, M. Baumann, B. Frangione, *Biochem. Biophys. Res. Commun.* **1996**, *226*, 672–680; j) J. Ghanta, C.-L. Shen, L. L. Kiessling, R. M. Murphy, *J. Biol. Chem.* **1996**, *271*, 29525–29528.

[6] a) C. Schmuck, M. Heil, *Org. Biomol. Chem.* **2003**, *1*, 633–636; b) C. Schmuck, M. Heil, *ChemBioChem* **2003**, *4*, 1232–1238.

[7] a) C. Schmuck, L. Geiger, *J. Am. Chem. Soc.* **2004**, *126*, 8898–8899; b) C. Schmuck, L. Geiger, *Curr. Org. Chem.* **2003**, *7*, 1485–1502; c) C. Schmuck, V. Bickert, *Org. Lett.* **2003**, *5*, 4579–4581; d) C. Schmuck, *Chem. Eur. J.* **2000**, *6*, 709–718; e) C. Schmuck, J. Lex, *Org. Lett.* **1999**, *1*, 1779–1781; f) C. Schmuck, *Chem. Commun.* **1999**, 843–844.

[8] a) M. R. Nilsson, *Methods* **2004**, *34*, 151–160; b) R. S. Boshuizen, J. P. M. Langeveld, M. Salmona, A. Williams, R. H. Meloen, J. P. M. Langedijk, *Anal. Biochem.* **2004**, *333*, 372–380; c) C. S. Goldbury, S. Wirtz, S. A. Müller, S. Sunderji, P. Wicki, U. Aebi, P. Frey, *J. Struct. Biol.* **2000**, *130*, 217–231.

[9] H. LeVine, *Protein Sci.* **1993**, *2*, 404–410.

[10] W. E. Klunk, R. F. Jacob, R. P. Mason, *Anal. Biochem.* **1999**, *266*, 66–76.

[11] a) S. W. Snyder, U. S. Ladror, W. S. Wade, G. T. Wang, L. W. Barrett, E. D. Matayoshi, H. J. Huffaker, G. A. Krafft, T. F. Holzman, *Biophys. J.* **1994**, *67*, 1216–1228; b) J. T. Jarrett, E. P. Berger, J. Peter T. Lansbury, *Biochemistry* **1993**, *32*, 4693–4697.

[12] C. Mambule, Y. Ando, I. Anan, G. Holmgren, O. Sandgren, T. Stigbrandt, K. Tashima, O. B. Suhr, *Biochim. Biophys. Acta* **2000**, *1474*, 331–336.

Received: July 29, 2004
Published online on February 18, 2005

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim