



Cite this: *Metallomics*, 2014, 6, 1931

Neuroprotective peptide–macrocycle conjugates reveal complex structure–activity relationships in their interactions with amyloid β

Mingfeng Yu,^a Timothy M. Ryan,^b Samantha Ellis,^c Ashley I. Bush,^b James A. Triccas,^c Peter J. Rutledge*^a and Matthew H. Todd*^a

Interactions between amyloid β (A β) and metal ions are thought to mediate the neuropathogenic effects of A β in Alzheimer's disease. The construction of small molecules capable of synergistically chelating metal ions and recognizing A β would allow new insights into the biology of this disease and provide a possible therapeutic approach. We report herein the synthesis and biological evaluation of tetraazamacrocyclic–(G)KLVFF hybrids and their metal complexes. The results obtained from ThT and bis-ANS extrinsic fluorescence assays, tyrosine intrinsic fluorescence assay and proteolytic assay imply complex, multifaceted structure–activity relationships in the interaction of these conjugates with A β . Many of the compounds tested rescued cells from A β -induced cytotoxicity. The attendant simplicity and ready diversification of the synthesis of these conjugates makes them attractive for further investigation.

Received 28th April 2014,
Accepted 31st July 2014

DOI: 10.1039/c4mt00122b

www.rsc.org/metallomics

Introduction

Alzheimer's disease (AD) is a progressive and multifactorial neurodegenerative disorder leading to the most common form of dementia in the elderly.^{1,2} The disease imposes a huge and growing burden on society.³ Progress towards understanding the underlying cause of AD has been made across a number of disciplines, yet its etiology and pathogenesis remain to be fully and precisely elucidated.⁴ Though it is not without its critics,^{5–8} the widely-supported amyloid hypothesis posits that aggregation of amyloid β (A β) and subsequent deposition into senile plaques (SPs) are involved in the progression of AD.⁹

Metal ions play an important role in the assembly of A β .^{1,10} Zinc(II)¹¹ and copper(II),¹² even at the trace (nanomolar) concentrations found in commonly-used laboratory buffers and culture media,¹³ induced marked A β aggregation *in vitro*. Elevated concentrations of copper, zinc and iron have been observed in SPs of AD patients.¹⁴ Redox-active copper(I/II) and iron(II/III) bound to A β can undergo Fenton-type chemistry to generate reactive oxygen species such as hydrogen peroxide (H₂O₂) and the hydroxyl

radical (\bullet OH),^{15,16} which can cause considerable oxidative damage to biological molecules and trigger neurodegeneration. These findings suggest that metal chelators could offer interesting new therapeutic benefits for AD by disrupting metal–A β interactions.

Current research efforts in this area centre on the construction of small molecules capable of synergistically chelating metal ions and recognizing A β .^{17,18} Such molecules consist of a metal chelator (*e.g.* clioquinol) and a known A β recognition group (*e.g.* curcumin, thioflavin-T). A particularly intriguing example of such a bifunctional molecule is the cyclen–pentapeptide hybrid **1** (Fig. 1A) in which cyclen is the metal chelator and the KLVFF peptide the A β recognition moiety.¹⁹ This hybrid was found to capture copper(II) bound to A β , become proteolytically active, interfere with A β oligomerization and aggregation, cleave A β into fragments, and prevent H₂O₂ formation and toxicity in neuronal cell culture. This precedent raises two interesting questions: (1) Is sequestration of the metal ion pivotal? (2) What structural elements are required for proteolysis to occur? Answering these questions may help to explain, among other things, why molecule **1** does not self-cleave. We became interested in exploring this structure for its possible therapeutic potential but also more broadly for the insights it might give to the behaviour of A β . We report herein the synthesis and biological evaluation of ten novel tetraazamacrocyclic–(G)KLVFF hybrids: the amide- or triazole-linked derivatives **2** and **4–6** (Fig. 1A) and their metal complexes **20**, **21** and **23–26** (Scheme 1).

These molecules were designed to provide insight into the structure–activity relationships that might be operating. Cyclam has a stronger binding affinity for copper(II) than does cyclen

^a School of Chemistry, The University of Sydney, Sydney, New South Wales 2006, Australia. E-mail: matthew.todd@sydney.edu.au, peter.rutledge@sydney.edu.au; Fax: +61 2 9351 3329; Tel: +61 2 9351 2180, +61 2 9351 5020

^b The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria 3010, Australia

^c Sydney Medical School, The University of Sydney, Sydney, New South Wales 2006, Australia

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4mt00122b



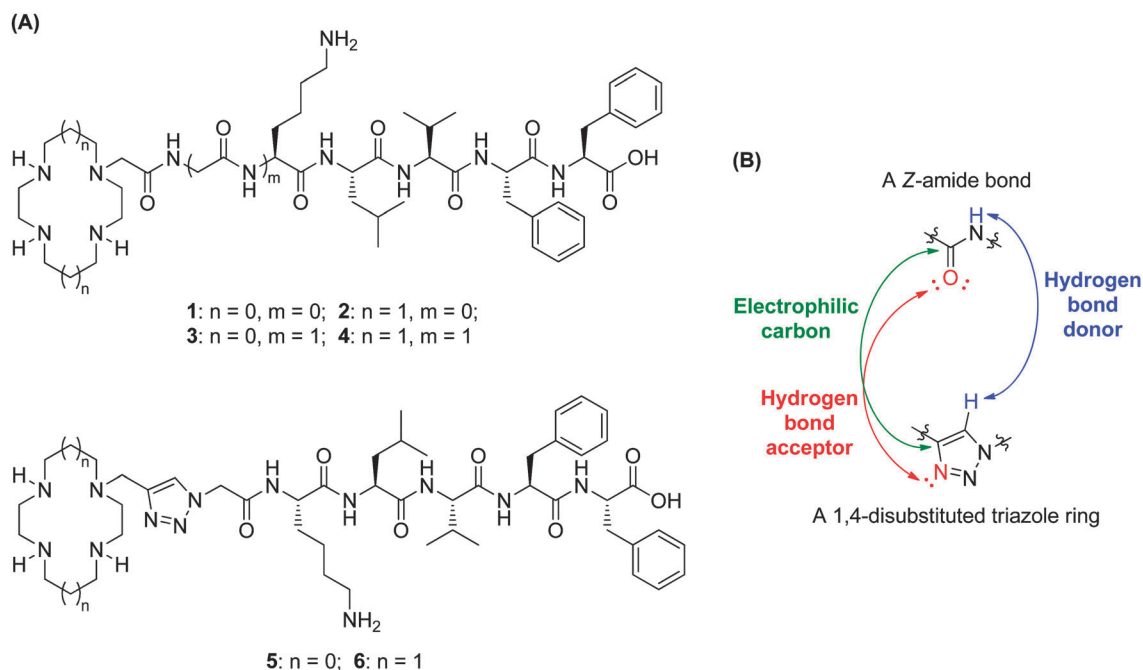


Fig. 1 (A) Structures of tetraazamacrocyclic-(G)KLVFF hybrids **1–6**; (B) electronic similarities between a Z-amide bond and a 1,4-disubstituted triazole ring.²⁰

($\log K$: 26.5 for copper(II)-cyclam vs. 23.3 for copper(II)-cyclen),²¹ suggesting that copper(II)-cyclam complexes are more likely to be formed *in vivo* than the corresponding copper(II)-cyclen complexes.²² Furthermore, the metal complexes of cyclen and cyclam tend to have different hydrolytic abilities, thus cyclam-KLVFF hybrid **2** was chosen to evaluate the role of the azamacrocyclic metal complex in any observed peptide cleavage. Synthetic pentapeptide KLVFF, a short A β fragment (A β _{16–20}), has been shown to bind full-length A β and disrupt its assembly into A β fibrils.^{23,24} To examine the importance of the length and nature of the recognition sequence, and its position relative to the metal centre, additional glycine spacers were introduced (hybrids **3** and **4**). The triazole moiety was incorporated into tetraazamacrocyclic-KLVFF hybrids to explore whether coordinative saturation of copper(II) with the additional triazole ligand affects proteolytic activity (hybrids **5** and **6**). Given that hybrids **5** vs. **3** and **6** vs. **4** are structurally identical except for the linker (triazole vs. amide), the biological potential of 1,4-disubstituted triazole as a Z-amide bioisostere²⁰ (Fig. 1B) could be assessed by comparing triazole-linked hybrids **5** and **6** with amide-tethered hybrids **3** and **4**. All six tetraazamacrocyclic-(G)KLVFF hybrids **1–6** and their corresponding copper(II) and zinc(II) complexes were synthesized and evaluated; the change in metal ion is of obvious interest to determine whether the specific nature of the metal complex is important in the biological potency of these conjugates.

Results and discussion

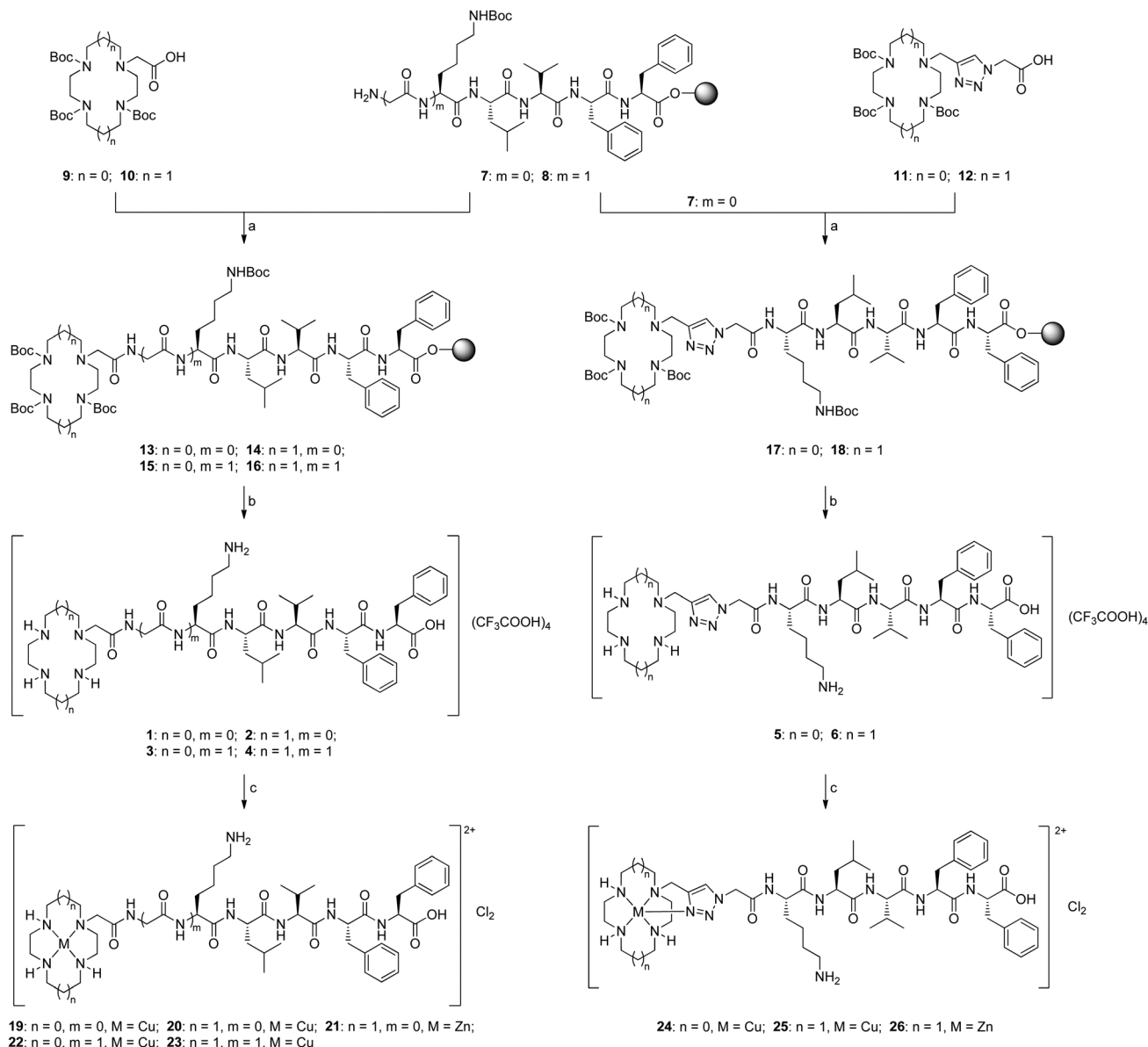
(a) Synthesis of tetraazamacrocyclic-(G)KLVFF hybrids and their metal complexes

The resin-bound oligopeptides KLVFF **7** and GKLFF **8** were assembled *via* Fmoc-strategy solid phase peptide synthesis

(SPPS) on Wang resin (Scheme S1, ESI[†]), and further coupled with tri-Boc-tetraazamacrocyclic-acetic acid **9** or **10** to give resin-bound Boc-protected tetraazamacrocyclic-(G)KLVFF compounds **13–16** (Scheme 1). Removal of Boc groups and detachment from the solid support were achieved in one pot by treatment of resin-bound compounds **13–16** with a cleavage/scavenger cocktail of TFA/TIS/H₂O (90 : 5 : 5). The crude products were purified by RP-HPLC and lyophilized to give the desired amide-tethered hybrids **1–4** as fluffy white solids in good overall yields. This solid-phase based synthetic procedure was successfully applied to the preparation of triazole-linked hybrids **5** and **6** by introducing tri-Boc-tetraazamacrocyclic-triazole-acetic acid **11** and **12** respectively to the resin-bound oligopeptide **7**. Elemental analysis data indicated that each of the isolated compounds **1–6** incorporated four equivalents of constitutive TFA (presumably three associated with secondary amino groups of the tetraazamacrocyclic and one with the ϵ -amino group of the lysine residue).

The trifluoroacetate salts of compounds **1–6** were complexed directly with copper(II) and zinc(II) as reported previously for related systems.^{25–27} Reaction with CuCl₂·2H₂O was carried out in EtOH at reflux for 6 hours to afford copper(II)-tetraazamacrocyclic complexes **19, 20** and **22–25**, which were isolated by centrifugation. The copper(II)-cyclen complexes appear blue whereas the copper(II)-cyclam complexes are purple powders (Fig. S1, ESI[†]). Zinc(II) complexes of hybrids **2** and **6** were also prepared by reacting the ligands with ZnCl₂ under similar conditions. Other metal cyclam-amino acid/peptide complexes that we have reported previously all exhibited characteristic singly charged cations in the high resolution mass spectra.²⁵ In contrast, all the metal complexes of tetraazamacrocyclic-(G)KLVFF hybrids gave rise exclusively to a cluster of [M–2Cl]²⁺ peaks with the correct isotope patterns. The purity of these metal complexes was confirmed by elemental





Scheme 1 Synthesis of tetraazamacrocyclic-(G)KLVFF hybrids **1–6** and their metal complexes **19–26**. Reagents and conditions: (a) appropriate carboxylic acid (**9**, **10**, **11** or **12**), PyBOP, NMM, DMF, rt, 1 h; (b) TFA/TIS/H₂O (90 : 5 : 5), rt, 2 h, followed by RP-HPLC purification, **1**: 53%, **2**: 63%, **3**: 52%, **4**: 60%, **5**: 60%, **6**: 58%; (c) CuCl₂·2H₂O or ZnCl₂, EtOH, reflux, 6 h, **19**: 94%, **20**: 81%, **21**: 54%, **22**: 85%, **23**: 88%, **24**: 67%, **25**: 53%, **26**: 69%.

analysis (ESI⁺). The UV-Vis spectra of the copper(II) complexes showed that λ_{max} and ϵ values associated with the cyclen complexes ($\lambda_{\text{max}} = 582\text{--}591$ nm, $\epsilon = 211\text{--}258$) were larger than those for the corresponding cyclam complexes ($\lambda_{\text{max}} = 552\text{--}555$ nm, $\epsilon = 110\text{--}138$). Absolute values of λ_{max} and ϵ are expected to be solvent-, pH- and substituent-dependent, but the same relationship of these values (cyclen > cyclam) has been observed previously,²⁸ as has the reverse relationship (cyclam > cyclen).²⁹ No significant differences in λ_{max} and ϵ values were observed between the triazole-linked complexes and the amide-tethered complexes.

(b) *In vitro* biological evaluation

With tetraazamacrocyclic-(G)KLVFF hybrids **1–6** and their metal complexes **19–26** in hand, a series of *in vitro* biological assays

was conducted using A β ₄₂ that included thioflavin-T (ThT) extrinsic fluorescence, tyrosine intrinsic fluorescence, bis-ANS extrinsic fluorescence and neurotoxicity assays, along with MALDI-TOF-MS analysis. Azide-capped pentapeptide **27** (see Scheme S1, ESI⁺ for the synthetic procedure) and the simple, non-peptidic metal-cyclam complexes **28** and **29**²⁵ were also evaluated as controls (Fig. 2). The fresh A β stock solution used for these studies was prepared by a modified literature procedure (ESI⁺).¹⁹ Pretreatment of A β with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) has been previously considered to be of benefit with respect to solubilizing the peptide and monomerizing β -sheet peptide aggregates, however this step was omitted because the alcohol has recently been shown to increase A β aggregation in solution.³⁰



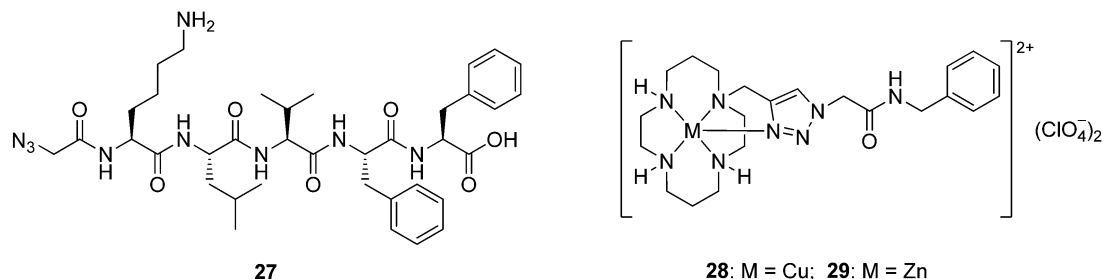


Fig. 2 Structures of three control compounds 27–29.

(i) **ThT extrinsic fluorescence assay.** The fluorescence emission maximum of ThT at 480–490 nm (excitation at 440–450 nm) is dramatically enhanced by its binding to A β fibrils.^{31,32} Accordingly, the fluorescence of ThT is widely used to quantify the inhibition of A β fibril formation in the presence of anti-amyloidogenic compounds *in vitro*.

A continuous ThT extrinsic fluorescence assay^{33,34} was employed to determine the effects of compounds 1–6 and 19–29 on A β aggregation. These compounds (10 and 50 μ M) were incubated with monomeric A β (5 μ M) and ThT (20 μ M) in PBS buffer (pH 7.4) for 23 hours, and the fluorescence of ThT was measured continuously throughout the incubation (excitation at 444 nm and emission at 485 nm). It was found that addition of the test compounds gave rise to (1) an increase (1, 3, 27 and 29), (2) a decrease (2, 6, 20, 21, 23, 24 and 26) or (3) little change (4, 5, 19, 22, 25 and 28) in the fluorescence intensity with comparison to that obtained from the case where monomeric A β alone was incubated with ThT (Fig. 3 and Fig. S2, ESI[†]), demonstrating that these compounds could (1) promote, (2) inhibit or (3) hardly affect the A β fibril formation respectively (Table 1).

Zinc(II) complexes 21 and 26 strongly inhibited formation of A β fibrils at both concentrations. The equivalent copper(II) complexes 20 and 25 respectively showed significantly reduced inhibitory activity: some activity was observed for 20 but only at high concentration, while no activity was observed for 25 at both concentrations. This result implies that the nature of the metal ion in such conjugates is an important factor in A β fibril inhibitory activity. The corresponding free ligands 2 and 6 had little effect on A β fibril formation at 50 μ M, but surprisingly exhibited inhibitory activity at 10 μ M. Copper(II) complexes 23 and 24 displayed modest activity against A β fibril formation at high concentration, while the corresponding free ligands 4 and 5 exerted little influence on A β aggregation. As ThT fluorescence is a spectroscopic measure of aggregation, and since it is known that the addition of compounds that are spectroscopically active can skew the results,³⁵ we confirmed these results using a pelleting assay, where the amount of aggregate formed over 24 hours was measured by the proportion of A β peptide that pelleted at 100 000 $\times g$ (Fig. S3, ESI[†]). The results showed that the ThT assay was measuring the proportion of aggregated A β accurately, confirming the results presented in Table 1. In addition, aggregation could be influenced by an interaction between the phosphate buffer and metal ions. We find no difference in the results when the PBS buffer is

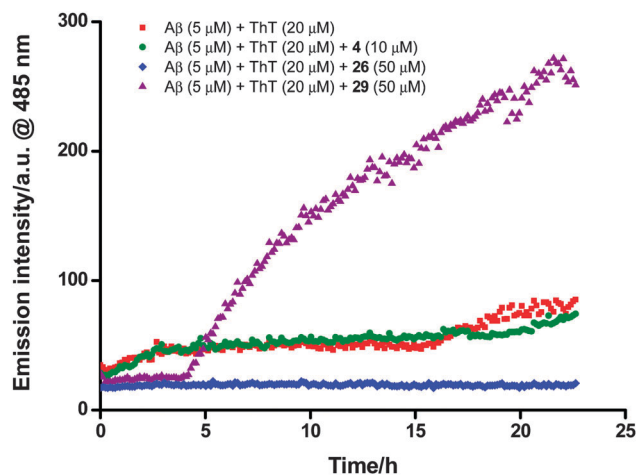


Fig. 3 Selected data from ThT extrinsic fluorescence assay: addition of the peptidic zinc(II) complex 26 (50 μ M) quenched the ThT fluorescence (blue diamond) with comparison to that obtained from an incubated solution of A β (5 μ M) and ThT (20 μ M) alone (red square), but the simple, non-peptidic zinc(II) complex 29 (50 μ M) enhanced the ThT fluorescence (purple up-pointing triangle). The free ligand 4 (10 μ M) exerted little influence on the ThT fluorescence (green circle).

Table 1 Effects of compounds 1–6 and 19–29 at concentrations of 10 and 50 μ M on A β aggregation

Compound	Effect on A β aggregation ^a	
	10 μ M	50 μ M
1	0	+++
2	---	0
3	+++	0
4	0	0
5	0	0
6	-	0
19	-	-
20	0	---
21	-	---
22	0	0
23	0	---
24	0	---
25	0	0
26	-	---
27	0	++
28	0	0
29	+	+++

^a (1) Inhibition: weak (-), moderate (---), strong (----); (2) no effect: 0; (3) promotion: weak (+), moderate (++), strong (+++).



substituted for Tris-HCl (20 mM, pH 7.5), suggesting that the PBS buffer is not interfering with the aggregation assay.

The copper(II)-cyclen complexes **19** and **22** had been reported to inhibit markedly the formation of ThT-positive A β aggregates.¹⁹ However, in the present study, these two complexes were found to have little effect on A β aggregation at either 10 or 50 μ M. The corresponding free amine ligands **1** and **3**, which differ only in the length of peptide sequences (KLVFF in **1** vs. GKLFFF in **3**), showed contrasting results: hybrid **1** significantly promoted the formation of A β fibrils at high concentration (but did not affect A β aggregation at low concentration), whereas compound **3** strongly activated A β fibril generation at low concentration (but had little effect on A β fibril formation at high concentration).

The peptide control **27** moderately accelerated A β fibril formation at high concentration. The simple (non-peptidic) zinc(II) complex **29** promoted the generation of A β fibril. In contrast, the equivalent copper(II) complex **28** showed no activity against A β fibril formation, providing further evidence for the importance of the nature of the metal ion.

The promotion of A β aggregation by compounds **1**, **3**, **27** and **29** was signalled by a dramatic increase in ThT fluorescence intensity during the incubation. It is conceivable that these compounds may somehow themselves aggregate to give a false positive, however incubation of each of compounds **1**, **3**, **27** and **29** with ThT in the absence of A β did not trigger any measurable fluorescence change (this control was performed for every compound used in this study, including the zinc(II) and copper(II) complexes, with the same result). This result confirmed that these compounds on their own do not form ThT-positive aggregates. There remains the possibility that ThT-positive heterofibrils are being formed between the added compounds and A β , which from a biochemical standpoint warrants further investigation.

These data reveal few if any obvious trends, making it difficult to develop a unified rationale for the effects observed. Overall, the peptidic zinc(II) complexes **21** and **26** exhibited the strongest A β fibril inhibitory activity among the test compounds whereas the simple, non-peptidic zinc(II) complex **29** was the only compound to accelerate A β fibril formation at both concentrations. None of the copper(II) complexes (**19**, **20**, **22–25** and **28**) promoted A β aggregation. Two of the unmetallated ligands (**2** and **6**) showed inhibitory activity against A β fibril formation at low concentration but hardly affected A β aggregation at high concentration, whereas the rest of them (**1** and **3–5**) either exerted little influence on or even strongly accelerated the formation of A β fibrils. There are no obvious patterns when comparing the different chelators (cyclam vs. cyclen), different spacers (*N*-benzylamide vs. KLVFF vs. GKLFFF), and different linkers (triazole vs. amide). The conclusion from this assay is that, rather than a simple A β :(G)KLVFF interaction giving a pre-defined outcome, there must be a complex combination of factors that control the interaction between these compounds and A β . It is possible that heterofibril assemblies^{36,37} form between the compounds and A β under the control of complex noncovalent interactions; while this is not equivalent to

inhibiting fibril formation, such assemblies could still be important from a medicinal chemistry perspective. Further work is required to investigate this possibility.

(ii) MALDI-TOF-MS. Given the established proteolytic activity of metal-cyclen complexes,^{19,38,39} MALDI-TOF-MS was carried out to evaluate the ability of the metal complexes **19–26** to cleave A β species; the free ligands **1–6** were also evaluated for comparison. Each compound was incubated with A β in PBS buffer (pH 7.4) for 4 and 7 days, and the resulting mixture desalted and analyzed by MALDI-TOF-MS. In all cases, the mass spectra showed no evidence for A β cleavage, suggesting that none of these compounds promote A β cleavage under the tested conditions. Metal complexes **19** and **22** had previously been reported to cleave A β species under similar conditions,¹⁹ but this was not observed in this study (Fig. S4, ESI[†]).

(iii) Tyrosine intrinsic fluorescence assay. Tyrosine intrinsic fluorescence of A β is quenched when copper(II) binds to the peptide and regained by addition of copper(II) chelators.^{19,40–42} This assay was used to explore whether tetraazamacrocyclic-(G)KLVFF hybrids **1–6** are capable of capturing copper(II) bound to A β . The corresponding metal complexes **19–26**, azide-capped pentapeptide **27** and the two simple metal-cyclam complexes **28** and **29** served as controls.

Excitation of A β (10 μ M) in Tris buffer (50 mM, pH 7.5) at 275 nm gave rise to the expected time-invariant maximum emission intensity at *ca.* 314 nm (Fig. 4A). This tyrosine fluorescence was quenched upon addition of copper(II) chloride (16 μ M) as previously reported,^{40,42} and subsequent incubation for 1 hour did not cause any further decrease (Fig. S5, ESI[†]). After the 1 hour incubation of A β with copper(II), the test compound (40 μ M) was added and co-incubated for a further hour. The total fluorescence of these three-component mixtures was measured, from which any intrinsic fluorescence of the test compounds themselves (measured separately – Fig. S5, ESI[†]) was subtracted. The resulting fluorescence intensity was compared with that obtained from the incubated mixture of A β and copper(II) alone to determine whether the test compounds could extract and capture copper(II) bound to A β (Fig. 4). Alternate additions of these compounds, either preloading the compounds with copper(II), or pre-incubating the A β with the compounds did not alter the results.

Hybrid **1** had previously been reported to reverse the copper(II)-induced quenching of A β tyrosine fluorescence due to sequestration of the metal ion from A β .¹⁹ However, in the present study, this free ligand exerted little effect on the tyrosine fluorescence of copper(II)-bound A β (Fig. 4B). Similar results were observed for the ligands **3**, **4** and **6**. In contrast, addition of ligands **2** and **5** resulted in the partial recovery of the tyrosine fluorescence, indicating a low to moderate ability to sequester copper(II) from A β .

Surprisingly, the tyrosine intrinsic fluorescence was partially restored upon incubation with several of the metal complexes (**21–24** and **26**). These compounds lack any obvious metal-chelating ability: the azamacrocyclic already holds a metal ion, and the peptide portion shows no demonstrable ability to sequester copper(II) under these conditions when tested as compound **27**. These results therefore imply that beyond (or perhaps instead of)



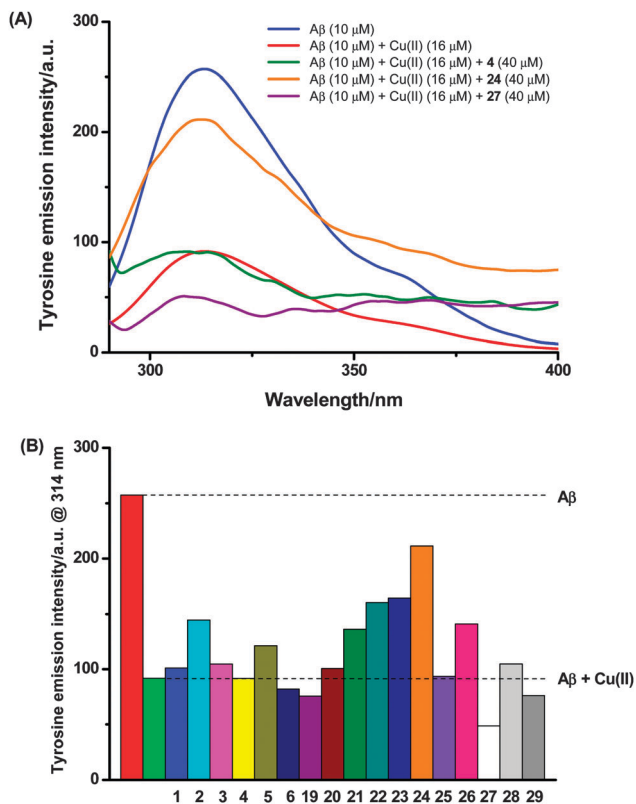


Fig. 4 (A) Selected data from tyrosine intrinsic fluorescence assay: tyrosine emission intensity of A β (10 μ M) in Tris buffer (50 mM, pH 7.5) at ca. 314 nm (blue) was quenched upon addition of copper(II) chloride (16 μ M) (red); subsequent incubation of the resulting mixture with compound **4**, **24**, or **27** (40 μ M) had little effect on (green), partially restored (orange), or further reduced (purple) the tyrosine intrinsic fluorescence respectively; (B) summary of the effects of compounds **1–6** and **19–29** on the copper(II)-induced quenching of A β tyrosine fluorescence.

the chelation effect, another, different interaction contributes to the revival of tyrosine fluorescence. The other compounds tested exert little or no influence on the tyrosine fluorescence. Compound **27** in fact further reduced the tyrosine fluorescence, which may be due to the oxidation of the tyrosine residue by the azido group.⁴³ As many of these compounds are coloured we cannot rule out some indirect influence on the tyrosine fluorescence through inner-filtering effects. Three points in our results suggest that this effect is not a significant factor: (i) the concentration of the compound, while fairly high, does not give rise to an OD greater than 0.1 arbitrary units at the excitation wavelength of tyrosine; (ii) only one uncoloured compound decreased the tyrosine fluorescence further, which, if inner filtering were significant, should have happened with more of the coloured compounds; and (iii) mixtures of A β and the compounds in the absence of copper(II) did not indicate any significant effect of either molecule on the fluorescence signal of the other, *i.e.* the total tyrosine fluorescence was simply the sum of the respective signals from the compound and A β .

Overall, more of the metal complexes were capable of restoring the tyrosine fluorescence than the free ligands, suggesting that alternative interaction(s) between metal complexes and

copper(II)-bound A β (*i.e.* other than the chelation and sequestration of copper), are responsible for this process. Thus we propose that the fluorescence is regained not through the chelation of copper, but possibly *via* the displacement of copper(II) from A β by the binding of these compounds. Previous deployment of this assay has used either glycine or histidine in large excess to bind copper^{40,42} rather than a discrete macrocyclic chelator to compete with A β for copper(II) as used here; it is plausible that in those cases too a binding interaction or displacement is occurring, rather than simple metal chelation as previously proposed. While the tyrosine fluorescence results observed in the present study would be explained by a more complex interaction between A β and the compounds tested, more work is required to validate this hypothesis. Such work is clearly important to confirm the meaning of this assay more generally.

(iv) **Bis-ANS extrinsic fluorescence assay.** The ability of compounds **1–6** and **19–29** to interfere with zinc(II) related enhancement of A β self-association was analyzed using the dye 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS). This dye has been shown to be particularly useful for the analysis of metal-A β interactions:^{44–46} the addition of a metal ion (zinc(II) in particular) causes a very large and persistent increase in the intensity of bis-ANS, which degrades over long timeframes as A β fibrils form.⁴⁷ We have shown that this intensity increase can be reversed by the addition of chelators, such as ethylenediaminetetraacetic acid (EDTA), suggesting the increase is due to the formation of a transient species stabilized by the presence of a metal ion (B. Roberts, Z. Datki and A. I. Bush, unpublished data). In the present study, we incubated A β in the presence of bis-ANS and compound, and then added zinc(II) to initiate the formation of bis-ANS positive A β oligomers. We conducted multiple controls to test for bis-ANS reactivity in the compounds alone and corrected all traces for any baseline we observed in these samples. We again see a diversity of effects of the added compounds on fibril formation ranging from (1) little effect (compound **26**), (2) partial inhibition (compounds **6**, **19**, **20**, **22–24** and **27–29**), and (3) complete inhibition (compounds **1–5**, **21** and **25**) (Fig. 5). In no case did we observe enhancement of the bis-ANS fluorescence (Fig. S6, ESI[†]), suggesting that these compounds did not enhance the formation of zinc(II)-induced partially folded and misfolded A β oligomers. The fact that compounds **1–6** inhibited the zinc(II) induced increase in bis-ANS fluorescence is unsurprising as these compounds are chelators and may thus act to compete with A β for the zinc(II), thereby removing the driving force for the formation of oligomeric species in the assay. However, the metallated complexes **19–25**, **28** and **29** should not have a chelating activity and thus a different mechanism must be invoked to explain the inhibition of the fluorescence intensity increase. One potential explanation is that the compounds interact with A β through a fairly specific binding interaction and either displace the bound zinc(II) or suppress the formation of bis-ANS positive oligomeric species of A β . As indicated for the tyrosine fluorescence assay, these results support this hypothesis and are interesting, but more work needs to be conducted to validate the presence of such an interaction and determine the consequent effects.



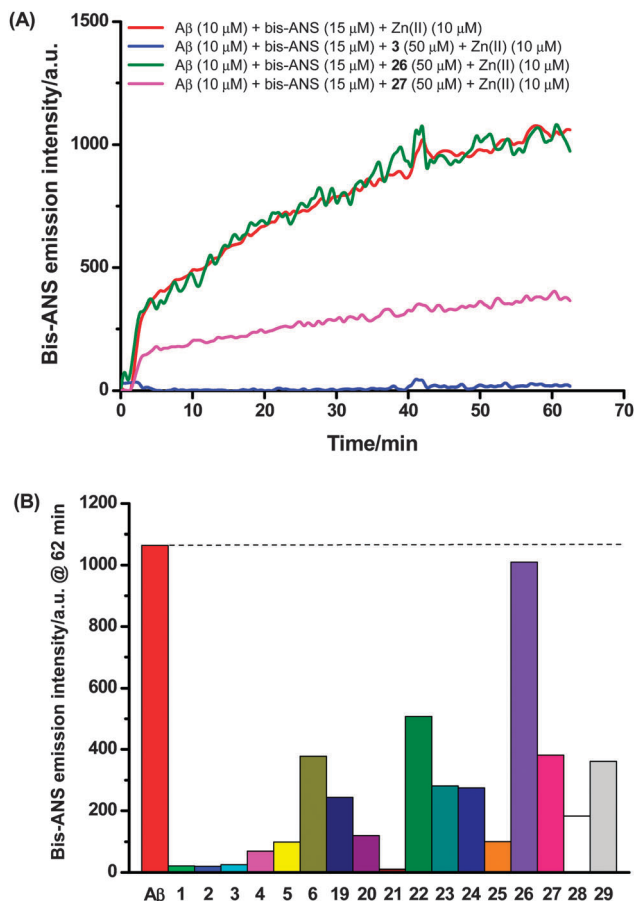


Fig. 5 (A) Selected data from bis-ANS extrinsic fluorescence assay: incubation of compound **3**, **26**, or **27** (50 μM) with A β (10 μM), bis-ANS (15 μM) and zinc(II) (10 μM) completely quenched (blue), exerted little influence on (green), or reduced (pink) the bis-ANS fluorescence respectively with comparison to that obtained from an incubated solution of A β (10 μM), bis-ANS (15 μM) and zinc(II) (10 μM) alone (red); (B) summary of the effects of compounds **1–6** and **19–29** on the bis-ANS fluorescence intensity at the end of the assay.

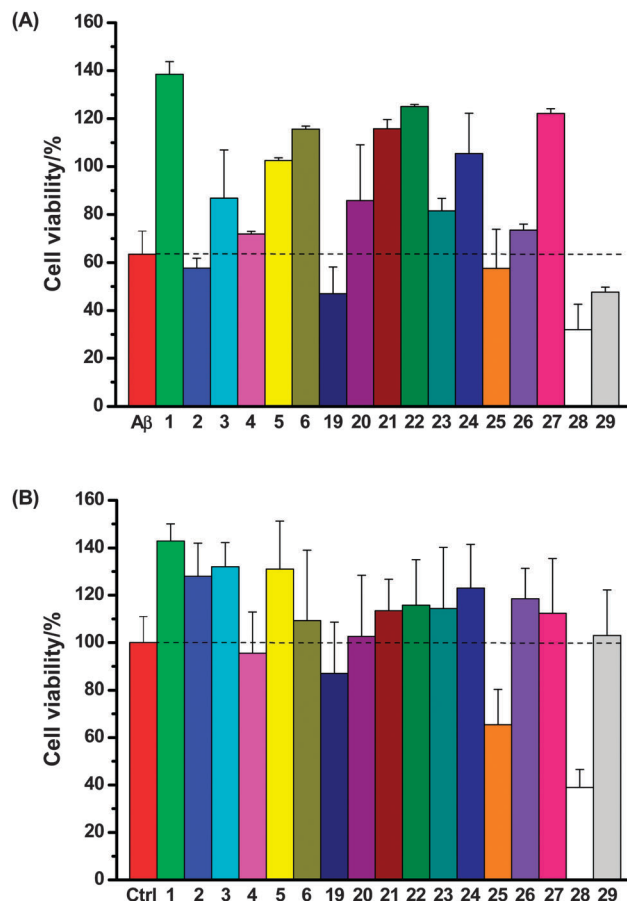


Fig. 6 (A) Effects of compounds **1–6** and **19–29** on A β -mediated toxicity in neuronal cell culture; (B) cytotoxicity of compounds **1–6** and **19–29** in neuronal cell culture in the absence of A β (Ctrl = SH-SY5Y neuron cells only (no test compound was added)).

25 and **28** themselves were cytotoxic (Fig. 6B), which correlates well with the heightened neurotoxicity observed with these compounds in the A β neurotoxicity assay.

(v) **Neurotoxicity assay.** Given the reported prevention of A β -mediated toxicity in neuronal cell culture by a cyclen-KLVFF hybrid,¹⁹ a neurotoxicity assay was conducted to examine whether compounds **1–6** and **19–29** could protect neurons from A β toxicity. First SH-SY5Y neuronal cells were incubated in the absence and presence of A β (0, 1, 5 and 10 μM) for 4 days. Neuronal viability was assessed by a Resazurin-based fluorescence assay, revealing that A β exhibited neurotoxicity only at 10 μM (Fig. S7, ESI[†]). Accordingly, this concentration was used in subsequent experiments.

SH-SY5Y neuronal cells were incubated with A β (10 μM) and the test compounds (50 μM) for 4 days; neuronal viability was then assessed using the same fluorescence assay (Fig. 6A). It was found that (1) most of these compounds (**1**, **3**, **5**, **6**, **20–24** and **27**) rescued SH-SY5Y neuronal cells from A β -induced neurotoxicity; (2) three compounds (**2**, **4** and **26**) exerted little protective effect on neurons; (3) four compounds marginally (**19**, **25**) or clearly (**28** and **29**) exacerbated the total neurotoxicity. Incubation of SH-SY5Y neuronal cells with the test compounds (50 μM) in the absence of A β suggested that only compounds **19**,

Experimental

Synthesis and characterization of tetraazamacrocyclic-(G)KLVFF hybrids and their metal complexes

See ESI[†] for complete experimental procedures and spectroscopic data.

A β_{42} stock solution preparation

A β_{42} (a lyophilized powder, the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University) (1 mg) was pre-treated with ammonium hydroxide as described previously.³⁰ The treated peptides were suspended in 60 mM NaOH (200 μL) and incubated for 5 min at room temperature. The resulting solution was diluted with Milli-Q water (700 μL) and sonicated at room temperature for a further 5 min in a water bath. The sonicated solution was neutralized with 10 \times PBS (PBS is defined as 50 mM sodium phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4, 100 μL) and centrifuged for 10 min at 14 000 $\times g$ in a



benchtop centrifuge. The optical density at 214 nm of the supernatant, containing the resolubilized A β ₄₂, was determined using a quartz microplate and a Flexstation 3 microplate reader (Molecular Devices) equipped with absorbance optics. The concentration was calculated from the 214 nm absorbance value using the molar extinction coefficient for A β ₄₂ of 94 530 M⁻¹ cm⁻¹.³⁴ Recovery of the peptide was typically 70–80%. The A β ₄₂ stock solution was immediately used for ThT extrinsic fluorescence assay, MALDI-TOF-MS, tyrosine intrinsic fluorescence assay, bis-ANS extrinsic fluorescence assay and neurotoxicity assay.

ThT extrinsic fluorescence assay

The effects of compounds **1–6** and **19–29** on the A β ₄₂ aggregation was evaluated using a continuous ThT fluorescence assay described previously.^{33,34} Compounds were dissolved in DMSO (Sigma Aldrich) to yield 1 mM stocks. These solutions were added into a 96-well microtitre plate (Wallac) containing ThT (Sigma Aldrich) and PBS (pH 7.4) to give final compound concentrations of 10 and 50 μ M with a ThT level of 20 μ M; A β ₄₂ was added to give a final concentration of 5 μ M. The plate was sealed with acetate adhesive seals (MP Biomedicals) to minimize evaporative loss, and incubated at 37 °C for 23 h. During incubation, the plate was shaken every 7 min for 3 s prior to the measurement of the ThT fluorescence intensity (excitation at 444 nm and emission at 485 nm) using a Flexstation 3 microplate reader (Molecular Devices).

A β ₄₂ pelleting assay

The results of the ThT assay were confirmed using a pelleting assay. Samples were prepared and treated as described for the ThT assay, and incubated at 37 °C for 24 h with shaking every 7 min for 3 s using an orbital plate shaker (350 rpm). 100 μ L of the sample was centrifuged at 100 000 \times *g* (Beckman Coulter, TL-100 benchtop ultracentrifuge), and the supernatant, pellet and starting material were assayed for protein concentration using a microBCA assay (Pierce) according to the manufacturer's instructions. The results were confirmed using the Direct Detect protein quantitation instrument from Millipore.

MALDI-TOF-MS

The proteolytic effects of compounds **1–6** and **19–29** on A β ₄₂ were investigated using MALDI-TOF-MS as described previously.¹⁹ The test compound (50 μ M) was incubated with or without A β ₄₂ (10 μ M) in PBS buffer (pH 7.4) for 4 and 7 days at 37 °C under sterile conditions. Each sample was desalted by a reverse phase C18 Zip-tip, and the resulting solution (1 μ L) was mixed 1:1 with matrix solution (10 mg mL⁻¹ α -cyano-4-hydroxycinnamic acid in a mixture of acetonitrile/water/TFA (50:49.9:0.1)) and spotted onto a ground steel target (MTP 384, Bruker Daltonics). Spotted samples were analyzed using a Bruker Daltonics Ultraflex III MALDI-TOF in reflector mode, with a detection range of 900–5000 Da, using appropriate peptide calibrants (Bruker Daltonics). Collected data were baseline corrected and smoothed using the Flexanalysis software module (Bruker Daltonics).

Tyrosine intrinsic fluorescence assay

Tyrosine intrinsic fluorescence assay^{19,40,42} was employed to investigate the effects of compounds **1–6** and **19–29** on the interaction between A β ₄₂ and copper(II). The tyrosine fluorescence spectra (excitation at 275 nm and emission at 290–305 nm) of A β ₄₂ (10 μ M) in Tris buffer (50 mM, pH 7.5) in a 96-well microtitre plate (Wallac) were acquired using a Flexstation 3 microplate reader (Molecular Devices) immediately after dissolution. After incubation for 1 h at 25 °C, fluorescence was measured again. Copper(II) chloride was added to give a final concentration of 16 μ M, and the fluorescence spectra of the resulting mixtures were acquired immediately. After incubation for 1 h at 25 °C, fluorescence was measured again. The test compounds (1 mM stocks in DMSO) were added to give a final concentration of 40 μ M, and the fluorescence spectra of the resulting mixtures were acquired immediately. After incubation for 1 h at 25 °C, fluorescence was measured again.

Bis-ANS extrinsic fluorescence assay

The ability of compounds **1–6** and **19–29** to interfere with zinc(II) related enhancement of A β self-association was analyzed using the dye 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS). The assay was conducted by adding bis-ANS, A β ₄₂ and compound to a microtitre plate containing 1 \times PBS (200 μ L) to final concentrations of 15, 10 and 50 μ M respectively at 25 °C. Upon mixing, the fluorescence emission was recorded at 485 nm with excitation at 390 nm using a Flexstation 3 Plate reader (Molecular Devices, Sunnyvale, California) at 1 minute intervals. After 5 minutes, zinc(II) was added to a final concentration of 10 μ M, and the fluorescence intensity was monitored for a further hour. All data were corrected for the intensity of changes of samples containing no A β ₄₂. Data reduction was done by taking the final point of the 60 minute time course.

Neurotoxicity assay

Neuronal cell line SH-SY5Y was cultured in RPMI (Invitrogen) supplemented with 10% heat inactivated FBS (Invitrogen), HEPES (25 mM; GIBCO), L-glutamine (100 mM; GIBCO) and β -mercaptoethanol. Cells were seeded at 1 \times 10⁵ cells per well of a 96-well microtitre plate (Falcon, BD Biosciences) in 100 μ L of culture media and allowed to adhere overnight under standard cell culture conditions (37 °C, 5% CO₂ and 95% humidity). Compounds **1–6** and **19–29** at a final concentration of 50 μ M in 0.1% DMSO (Sigma-Aldrich) were combined with A β ₄₂ at final concentrations of 1, 5 and 10 μ M in triplicate in appropriate wells. Cells, media and A β ₄₂ alone were used as controls. The plates were incubated for 96 h (37 °C, 5% CO₂ and 95% humidity). Resazurin (Sigma-Aldrich) was added at a final concentration of 0.05% (w/v) per well and incubated for 4 h before a measurement of fluorescent intensity was recorded on a FLUOstar OMEGA at 540/595 nm. Addition of compounds incorporating zinc(II) or copper(II) directly to resazurin did not alter the output fluorescence after 4 days incubation at 37 °C in 5% CO₂. The percentage cell viability was calculated in relation to the maximum and minimum measurement of fluorescence



caused by cells only (100% survival) or media alone (100% inhibition). All experiments were performed in duplicate, $n = 2$.

Conclusions

Tetraazamacrocyclic-(G)KLVFF hybrids **1–6** were prepared efficiently in good overall yields using a solid-phase based synthetic procedure. Complexation of these six conjugates with zinc(II) and copper(II) salts proceeded smoothly to give the corresponding metal complexes **19–26** in good to excellent yields. The biological activities of these tetraazamacrocyclic-(G)KLVFF hybrids and their metal complexes were evaluated *in vitro* using several assays. Both the extrinsic fluorescence assays using ThT and bis-ANS, and the tyrosine intrinsic fluorescence assay imply complex, multifaceted structure–activity relationships in the interaction of these conjugates and A β . None of the compounds tested degrade A β into fragments. However most of these compounds protect neurons from A β toxicity.

The previously reported ability of compound **1** to capture copper(II) ions and become proteolytically active could not be replicated in the present study. The differences might be explained by the different sources of A β or their slightly different methods of preparation. In the previous report, the A β was prepared in HFIP, but this was not used here as it has been found that HFIP can increase A β aggregation on its own.³⁰ While it is known that A β prepared with alternative pretreatments to HFIP, such as ammonium hydroxide (NH₄OH), differs in its aggregation speed, NH₄OH pretreatment was used in the present work since our previous work shows that this treatment results in an almost monomeric starting solution.³⁰ The methods of preparation of the metal complexes also differed: in the present work the metal complexes were isolated and purified, whereas in the previous work the complexes were prepared *in situ* with a slight excess of the ligand over the metal (1.2 : 1). The association constants for ligands/metal ions of this general class are high,^{48–51} meaning one would expect a vanishingly small free metal ion concentration from a 1 : 1 mixture of metal and ligand, but the slight excess of the free ligand used in the previous work could mean it is possible there was some free ligand present in the sample when it was biologically evaluated.

More generally for the overall set of compounds described in this paper, the results do not clearly articulate any consistent SARs and instead the data – particularly for the pre-formed metal complexes – suggest that interactions between compounds of this class and A β are complex. This is not unexpected given the *a priori* complexity of any binding interaction and what has been learned from the recent use of simple non-metallated peptides in the inhibition of amyloid formation.^{52–58} The results from the tyrosine intrinsic fluorescence assay in particular should caution that the changes in optical output observed using this assay may be caused by more complex changes than simple metal sequestration (which certainly should be more likely with the free ligands tested than the pre-formed metal complexes, in contrast with the outcomes discussed above). Further work is needed to verify whether other peptide- or chelator–A β interactions can give rise to the observed

fluorescence quenching. Despite the lack of clear patterns and SARs from the fluorescence and proteolytic assays with isolated protein, many of the compounds screened demonstrated an impressive ability to rescue cells from A β -induced cytotoxicity. The attendant simplicity and ready diversification of their synthesis makes this a promising class of compounds for further investigation.

Acknowledgements

We thank the National Breast Cancer Fund for a Novel Concept Grant (NC-10-69) and the Australian Research Council for funding (DP120104035). M. Yu was supported by a University of Sydney International Scholarship (USyDIS). T. M. Ryan is on a fellowship with the Australia Alzheimer's Dementia Research Foundation (AADRF).

References

- 1 P. A. Adlard and A. I. Bush, *J. Alzheimer's Dis.*, 2006, **10**, 145–163.
- 2 A. Bush and R. Tanzi, *Neurotherapeutics*, 2008, **5**, 421–432.
- 3 M. D. Hurd, P. Martorell, A. Delavande, K. J. Mullen and K. M. Langa, *N. Engl. J. Med.*, 2013, **368**, 1326–1334.
- 4 R. Jakob-Roetne and H. Jacobsen, *Angew. Chem., Int. Ed.*, 2009, **48**, 3030–3059.
- 5 A. D. Korczyn, *Alzheimer's Dementia*, 2008, **4**, 176–178.
- 6 C. Reitz, *Int. J. Alzheimer's Dis.*, 2012, 369808, DOI: 10.1155/2012/369808.
- 7 R. A. Armstrong, *Int. J. Alzheimer's Dis.*, 2011, 630865, DOI: 10.4061/2011/630865.
- 8 S. W. Pimplikar, *Int. J. Biochem. Cell Biol.*, 2009, **41**, 1261–1268.
- 9 J. A. Hardy and G. A. Higgins, *Science*, 1992, **256**, 184–185.
- 10 P. Faller, *ChemBioChem*, 2009, **10**, 2837–2845.
- 11 A. I. Bush, W. H. Pettingell, G. Multhaup, M. d. Paradis, J. P. Vonsattel, J. F. Gusella, K. Beyreuther, C. L. Masters and R. E. Tanzi, *Science*, 1994, **265**, 1464–1467.
- 12 C. S. Atwood, R. D. Moir, X. Huang, R. C. Scarpa, N. M. E. Bacarra, D. M. Romano, M. A. Hartshorn, R. E. Tanzi and A. I. Bush, *J. Biol. Chem.*, 1998, **273**, 12817–12826.
- 13 X. Huang, C. S. Atwood, R. D. Moir, M. A. Hartshorn, R. E. Tanzi and A. I. Bush, *J. Biol. Inorg. Chem.*, 2004, **9**, 954–960.
- 14 M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell and W. R. Markesbery, *J. Neurol. Sci.*, 1998, **158**, 47–52.
- 15 X. Huang, C. S. Atwood, M. A. Hartshorn, G. Multhaup, L. E. Goldstein, R. C. Scarpa, M. P. Cuajungco, D. N. Gray, J. Lim, R. D. Moir, R. E. Tanzi and A. I. Bush, *Biochemistry*, 1999, **38**, 7609–7616.
- 16 X. Huang, M. P. Cuajungco, C. S. Atwood, M. A. Hartshorn, J. D. A. Tyndall, G. R. Hanson, K. C. Stokes, M. Leopold, G. Multhaup, L. E. Goldstein, R. C. Scarpa, A. J. Saunders, J. Lim, R. D. Moir, C. Glabe, E. F. Bowden, C. L. Masters, D. P. Fairlie, R. E. Tanzi and A. I. Bush, *J. Biol. Chem.*, 1999, **274**, 37111–37116.



- 17 C. Hureau, I. Sasaki, E. Gras and P. Faller, *ChemBioChem*, 2010, **11**, 950–953.
- 18 J. J. Braymer, A. S. DeToma, J. S. Choi, K. S. Ko and M. H. Lim, *Int. J. Alzheimer's Dis.*, 2011, 623051, DOI: 10.4061/2011/623051.
- 19 W. Wu, P. Lei, Q. Liu, J. Hu, A. P. Gunn, M. Chen, Y. Rui, X. Su, Z. Xie, Y. Zhao, A. I. Bush and Y. Li, *J. Biol. Chem.*, 2008, **283**, 31657–31664.
- 20 G. C. Tron, T. Pirali, R. A. Billington, P. L. Canonico, G. Sorba and A. A. Genazzani, *Med. Res. Rev.*, 2008, **28**, 278–308.
- 21 V. J. Thom, G. D. Hosken and R. D. Hancock, *Inorg. Chem.*, 1985, **24**, 3378–3381.
- 22 S. J. Paisey and P. J. Sadler, *Chem. Commun.*, 2004, 306–307.
- 23 M. M. Pallitto, J. Ghanta, P. Heinzelman, L. L. Kiessling and R. M. Murphy, *Biochemistry*, 1999, **38**, 3570–3578.
- 24 L. O. Tjernberg, J. Näslund, F. Lindqvist, J. Johansson, A. R. Karlström, J. Thyberg, L. Terenius and C. Nordstedt, *J. Biol. Chem.*, 1996, **271**, 8545–8548.
- 25 M. Yu, J. R. Price, P. Jensen, C. J. Lovitt, T. Shelper, S. Duffy, L. C. Windus, V. M. Avery, P. J. Rutledge and M. H. Todd, *Inorg. Chem.*, 2011, **50**, 12823–12835.
- 26 M. Yu, Q. Yu, P. J. Rutledge and M. H. Todd, *ChemBioChem*, 2013, **14**, 224–229.
- 27 M. Yu, N. H. Lim, S. Ellis, H. Nagase, J. A. Triccas, P. J. Rutledge and M. H. Todd, *ChemistryOpen*, 2013, **2**, 99–105.
- 28 L. M. P. Lima, D. Esteban-Gómez, R. Delgado, C. Platas-Iglesias and R. Tripier, *Inorg. Chem.*, 2012, **51**, 6916–6927.
- 29 H. Ozay, Y. Baran and Y. Ishii, *Spectrochim. Acta, Part A*, 2011, **83**, 525–531.
- 30 T. M. Ryan, J. Caine, H. D. T. Mertens, N. Kirby, J. Nigro, K. Breheny, L. J. Waddington, V. A. Streltsov, C. Curtain, C. L. Masters and B. R. Roberts, *PeerJ*, 2013, **1**, e73.
- 31 A. Hawe, M. Sutter and W. Jiskoot, *Pharm. Res.*, 2008, **25**, 1487–1499.
- 32 H. Naiki, K. Higuchi, M. Hosokawa and T. Takeda, *Anal. Biochem.*, 1989, **177**, 244–249.
- 33 T. M. Ryan, A. Friedhuber, M. Lind, G. J. Howlett, C. Masters and B. R. Roberts, *J. Biol. Chem.*, 2012, **287**, 16947–16954.
- 34 G. McColl, B. R. Roberts, A. P. Gunn, K. A. Perez, D. J. Tew, C. L. Masters, K. J. Barnham, R. A. Cherny and A. I. Bush, *J. Biol. Chem.*, 2009, **284**, 22697–22702.
- 35 S. A. Hudson, H. Ecroyd, T. W. Kee and J. A. Carver, *FEBS J.*, 2009, **276**, 5960–5972.
- 36 B. O'Nuallain, A. D. Williams, P. Westermark and R. Wetzel, *J. Biol. Chem.*, 2004, **279**, 17490–17499.
- 37 C. J. Sarell, P. G. Stockley and S. E. Radford, *Prion*, 2013, **7**, 359–368.
- 38 J. Suh, S. H. Yoo, M. G. Kim, K. Jeong, J. Y. Ahn, M. S. Kim, P. S. Chae, T. Y. Lee, J. Lee, J. Lee, Y. A. Jang and E. H. Ko, *Angew. Chem., Int. Ed.*, 2007, **46**, 7064–7067.
- 39 J. Suh, *Acc. Chem. Res.*, 2003, **36**, 562–570.
- 40 C. D. Syme, R. C. Nadal, S. E. J. Rigby and J. H. Viles, *J. Biol. Chem.*, 2004, **279**, 18169–18177.
- 41 W. Garzon-Rodriguez, A. K. Yatsimirsky and C. G. Glabe, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2243–2248.
- 42 Q. F. Ma, J. Hu, W. H. Wu, H. D. Liu, J. T. Du, Y. Fu, Y. W. Wu, P. Lei, Y. F. Zhao and Y. M. Li, *Biopolymers*, 2006, **83**, 20–31.
- 43 V. Kadlcik, C. Sicard-Roselli, T. A. Mattioli, M. Kodicek and C. Houée-Levin, *Free Radical Biol. Med.*, 2004, **37**, 881–891.
- 44 W.-T. Chen, Y.-H. Liao, H.-M. Yu, I. H. Cheng and Y.-R. Chen, *J. Biol. Chem.*, 2011, **286**, 9646–9656.
- 45 W.-T. Chen, C.-J. Hong, Y.-T. Lin, W.-H. Chang, H.-T. Huang, J.-Y. Liao, Y.-J. Chang, Y.-F. Hsieh, C.-Y. Cheng, H.-C. Liu, Y.-R. Chen and I. H. Cheng, *PLoS One*, 2012, **7**, e35807.
- 46 B. R. Roberts, T. M. Ryan, A. I. Bush, C. L. Masters and J. A. Duce, *J. Neurochem.*, 2012, **120**, 149–166.
- 47 I. I. H. LeVine, *Arch. Biochem. Biophys.*, 2002, **404**, 106–115.
- 48 Y. H. Lau, J. R. Price, M. H. Todd and P. J. Rutledge, *Chem. – Eur. J.*, 2011, **17**, 2850–2858.
- 49 E. Tamanini, A. Katewa, L. M. Sedger, M. H. Todd and M. Watkinson, *Inorg. Chem.*, 2009, **48**, 319–324.
- 50 E. Tamanini, K. Flavin, M. Motevalli, S. Piperno, L. A. Gheber, M. H. Todd and M. Watkinson, *Inorg. Chem.*, 2010, **49**, 3789–3800.
- 51 K. Jobe, C. H. Brennan, M. Motevalli, S. M. Goldup and M. Watkinson, *Chem. Commun.*, 2011, **47**, 6036–6038.
- 52 M. A. Findeis, G. M. Musso, C. C. Arico-Muendel, H. W. Benjamin, A. M. Hundal, J.-J. Lee, J. Chin, M. Kelley, J. Wakefield, N. J. Hayward and S. M. Molineaux, *Biochemistry*, 1999, **38**, 6791–6800.
- 53 T. L. Lowe, A. Strzelec, L. L. Kiessling and R. M. Murphy, *Biochemistry*, 2001, **40**, 7882–7889.
- 54 S. M. Chafekar, H. Malda, M. Merckx, E. W. Meijer, D. Viertel, H. A. Lashuel, F. Baas and W. Scheper, *ChemBioChem*, 2007, **8**, 1857–1864.
- 55 M. Convertino, A. Vitalis and A. Caflisch, *J. Biol. Chem.*, 2011, **286**, 41578–41588.
- 56 T. Härd and C. Lendel, *J. Mol. Biol.*, 2012, **421**, 441–465.
- 57 S. A. Sievers, J. Karanicolas, H. W. Chang, A. Zhao, L. Jiang, O. Zirafi, J. T. Stevens, J. Munch, D. Baker and D. Eisenberg, *Nature*, 2011, **475**, 96–100.
- 58 C. T. Middleton, P. Marek, P. Cao, C.-c. Chiu, S. Singh, A. M. Woys, J. J. de Pablo, D. P. Raleigh and M. T. Zanni, *Nat. Chem.*, 2012, **4**, 355–360.

