Carboxymethylated-κ-casein: A convenient tool for the identification of polyphenolic inhibitors of amyloid fibril formation

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ABSTRACT

Reduced and carboxymethylated-κ-casein (RCM-κ-CN) is a milk-derived amyloidogenic protein that readily undergoes nucleation-dependent aggregation and amyloid fibril formation via a similar pathway to disease-specific amyloidogenic peptides like amyloid beta (Aβ), which is associated with Alzheimer's disease. In this study, a series of flavonoids, many known to be inhibitors of Aβ fibril formation, were screened for their ability to inhibit RCM-κ-CN fibrilisation, and the results were compared with literature data on Aβ inhibition. Flavonoids that had a high degree of hydroxylation and molecular planarity gave good inhibition of RCM-κ-CN fibril formation. IC_{50} values were between 10- and 200-fold higher with RCM-κ-CN than literature results for Aβ fibril inhibition, however, with few exceptions, they showed a similar trend in potency. The convenience and reproducibility of the RCM-κ-CN assay make it an economic alternative first screen for Aβ inhibitory activity, especially for use with large compound libraries.

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1. Introduction

Alzheimer's disease (AD) is a common and debilitating neurodegenerative disorder characterised by gradual loss of cognitive function and behavioural abnormalities.[1] One of the pathological hallmarks of AD is amyloid fibrillar deposits in the brain that consist mainly of the β-amyloid peptides $Aβ_{1-40}$ and $Aβ_{1-42}$.[2] These peptides are proteolytic fragments of the amyloid precursor protein and are highly hydrophobic and prone to aggregation. Although Aβ is normally present as a random coil, it can form intermediate oligomers that misfold into fibrils which aggregate to form insoluble amyloid plaques. The mechanism for amyloid formation is postulated to occur through a nucleation-dependent polymerisation.[3]

Since the formation of Aβ fibrillar assemblies is implicated in AD pathogenesis, food components and therapeutic agents capable of suppressing or preventing the transition from monomeric to oligomeric and polymeric Aβ species are attractive for the prevention or treatment of AD. Several small molecules have been reported to inhibit the aggregation of Aβ, and have been proposed as therapeutic drug candidates for this disease.[4] In relation to this, there are currently at least two low molecular weight compounds that target Aβ oligomerisation in clinical trials for the treatment of AD; a synthetic derivative of a natural product isolated from the South American rainforest vine, *Uncaria tomentosa*,[5] and scyllo-inositol.[6]

The discovery of novel inhibitors of Aβ aggregation is hampered by the limitations of existing screening methods.[7] Typically synthetic Aβ peptides are used; however, the preparation of Aβ is difficult and costly because it readily aggregates. This propensity to aggregate can also interfere with the efficacy of a screen because synthetic Aβ often contains oligomeric 'seeds' that can nucleate further aggregation. The use of synthetic Aβ in the screening of large compound libraries thus lacks practicality, and its cost makes such screening prohibitive for most laboratories. Alternatives need to be found.

The ability of polypeptide chains to form amyloid fibrils is not restricted to Aβ, but appears to represent a generic feature of aggregated polypeptide chains.[8] These amyloid structures share common characteristics such as unbranched fibrillar morphology, cross β-sheet structure and binding of the amyloid-specific dye thioflavin T (ThT). In recent years we have been studying the properties of a derivative of κ-casein, a major protein found in bovine milk, which displays an inherent propensity to rapidly form amyloid fibrils when incubated under conditions of physiological pH and temperature, with no requirement for denaturants.[9] This derivative of κ-casein (denoted as RCM-κ-CN) is readily prepared by reduction of the κ-casein disulfide linkages and subsequent carboxymethylation. Like Aβ fibrils, amyloid fibrils formed from RCM-κ-CN are cytotoxic.[10] Fibril formation by RCM-κ-CN can be easily

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monitored using an in situ ThT fluorescence binding assay similar to that used in assays that study the fibrilisation of Aβ.[11] The mechanism of such fibril formation by RCM-κ-CN has recently been studied in detail,[12] and this protein has shown itself to be a convenient model with which to study generic aspects of fibril formation.

Flavonoid compounds commonly found in tea and wine have demonstrated a remarkable propensity to inhibit the formation and associated toxicity of Aβ fibrillar assemblies in vitro. For example, the most abundant polyphenolic extract from green tea, (−)-epigallocatechin-3-gallate (EGCG, **1**, Fig. 1), is one of the most potent inhibitors of amyloid fibril formation of Aβ, huntington and α-synuclein,[13] the amyloidogenic proteins associated with Alzheimer's, Huntington's and Parkinson's diseases, respectively. Very recently we showed that EGCG also efficiently inhibits fibril formation by RCM-κ-CN and protects pheochromocytoma-12 cells from RCM-κ-CN amyloid-induced toxicity.[10]

Given the difficulties typically encountered with the screening of small molecules against synthetic Aβ, it was decided that RCM-κ-CN be investigated for its potential to serve as a convenient initial screen for small molecule Aβ anti-fibrilisation activity. Herein are described the results of an investigation of the ability of a set of common polyphenolics and their derivatives to inhibit fibril formation by RCM-κ-CN, as measured by the in situ ThT fluorescence binding assay. The findings have been compared with literature Aβ anti-fibrilisation data that were obtained using very similar assay methods.

2. Results

Examples of catechins (Fig. 1), flavonols (Fig. 2) and isoflavones (Fig. 3) and closely related compounds were chosen for study because of their well-known abilities to inhibit Aβ fibril formation. The specific examples were selected so that preliminary structure–activity relationships (SAR) in the inhibition of RCM-κ-CN fibril formation could be developed. All of the compounds used in this study are either commercially available, can be readily prepared from commercial starting materials, or in the case of (−)-epiafzelechin (**4**), conveniently isolated from a natural source. They were assayed using similar methods to those recently described,[10] such that the test compounds were incubated with RCM-κ-CN and ThT at physiological pH and temperature in a

fluorescence plate reader. In this study, a small amount of DMSO (1% v/v) was used to solubilise the test compounds and controls showed that this amount did not affect the assay. Under the conditions of the assay, the fluorescence of ThT increases in proportion to the amount of fibrillar protein present. After fibril formation was complete, usually after 1000 min, the intensity of fluorescence at 490 nm was measured and compared to a control that lacked the test compound. The difference in final fluorescence measured in the control and in the presence of the test compound was used to calculate % inhibition of fibril formation (%FFi, Table 1). In cases where the % inhibition was high (usually 80% or higher), a dose–response curve was recorded and from that, an IC_{50} value was determined. Only the inhibition of fibril formation by EGCG (**1**) with RCM-κ-CN has been previously reported. The IC_{50} value for **1** determined here is lower than that previously reported[10] because the concentration of RCM-κ-CN used in the present study was approximately half of that used by Hudson et al.[10]

It is assumed that ThT fluorescence is reduced in the presence of certain test compounds because these additives disrupt RCM-κ-CN fibril formation. There are, however, a number of other explanations for such an observation which are unrelated to FFi including; displacement of ThT from the growing fibrils without fibril disruption, interference with ThT fluorescence through physical association of the test compound within the growing fibrils, or light absorption by the test compound in either the excitation or emission frequencies of ThT.[20] To confirm that the compounds that gave the lowest IC_{50} values in the ThT assay did actually prevent fibril formation, transmission electron microscopy (TEM) images were recorded of a selection of post-assay mixtures (Fig. 4). Such corroborative evidence has already been established with the most active compound (**1**).[10] Gratifyingly, the micrographs in Figure 4 show a good correlation between IC_{50} values and the number of fibrils observed in the TEM images. Where test compounds indicated a strong inhibition in the ThT assay ($IC_{50} \leqslant 14$ μM; **6**, **8**, **11** and **13**) very few fibrils were observed in the corresponding TEM image compared to the control mixture, which contained 1% DMSO but no other additive. In addition, the fibrils in these samples were shorter and/or stunted. Test compounds that gave slightly higher IC_{50} values in the ThT assay (**7**, **9**), although allowing much fewer fibrils to grow than in the control sample, permitted more fibrils than compounds **6**, **8**, **11** and **13**.
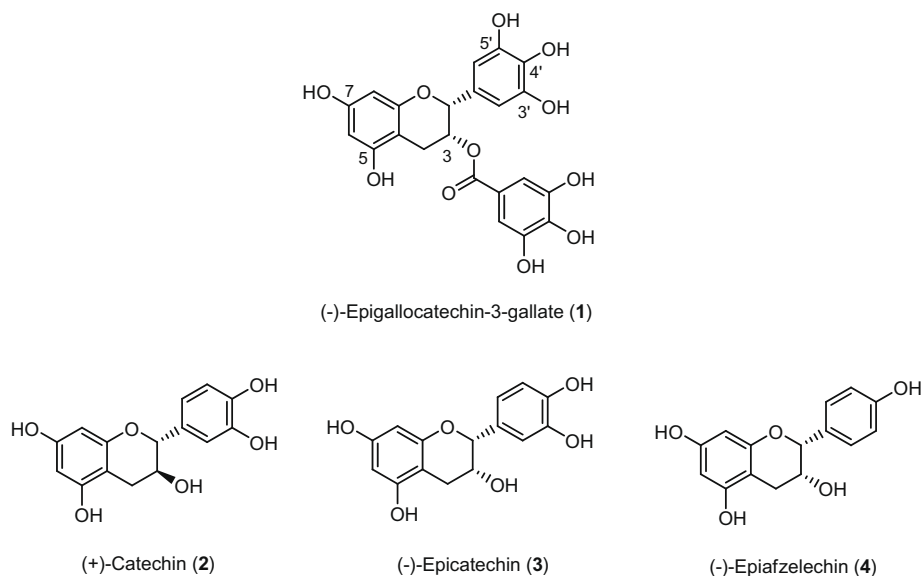

(-)-Epigallocatechin-3-gallate (**1**)

(+)-Catechin (**2**)   (-)-Epicatechin (**3**)   (-)-Epiafzelechin (**4**)

Figure 1. Catechins tested for their ability to inhibit RCM-κ-CN fibril formation.
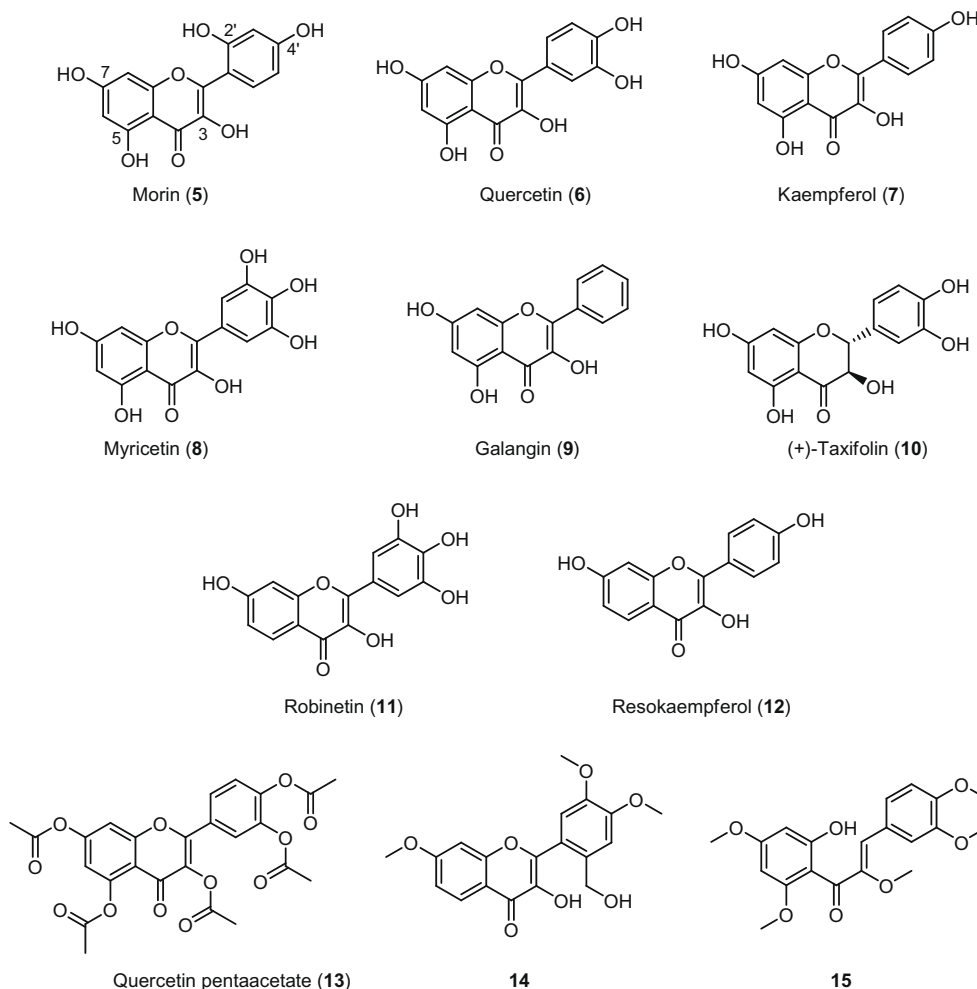
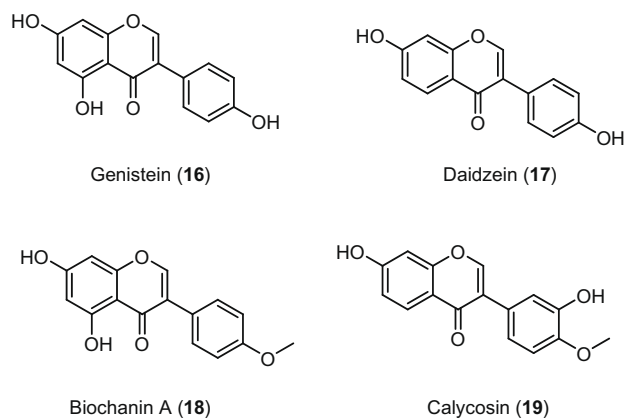**Figure 2.** Flavonols and related compounds tested for their ability to inhibit RCM-κ-CN fibril formation.

Figure 3. Isoflavones tested for their ability to inhibit RCM-κ-CN fibril formation.

3. Discussion

3.1. SAR in the inhibition of RCM-κ-CN fibril formation

A major criterion used to select compounds employed in this study was their suitability to identify the main SAR relationships in the flavonoid class of compounds in the RCM-κ-CN assay. The compounds shown in Figure 1 were chosen so SAR

information around the catechin class of compounds could be obtained, particularly in relation to the highly active catechin, EGCG (**1**). The largest set of closely related compounds examined was the flavonol group shown in Figure 2 (**5–15**). These compounds were chosen so that the effect of key structural features such as hydroxy substitution patterns, methylation and acetylation of the hydroxy groups, and the importance of molecular planarity, on FFi activity could be gauged. The isoflavones (Fig. 3) were chosen because of their close structural similarity with flavonols.

One of the most active compounds tested was myricetin (**8**, $IC_{50} = 8.5 \pm 0.7$ μM), which is the most highly hydroxylated flavonol in the set. The removal of just one hydroxy group from **8**, at the 5′-position, led to a measureable drop in activity (**6**, $IC_{50} = 13.6 \pm 3.8$ μM) and further deletion of the remaining 3′- and 4′-hydroxy groups led to a progressive loss in activity (**7**, $IC_{50} = 33.0 \pm 2.6$ μM; **9**, $IC_{50} = 61 \pm 44$ μM). The removal of the 5-hydroxy group on the chromen-4-one ring system again led to a drop in activity (**11**, $IC_{50} = 13.9 \pm 9.7$ μM; **12**, no activity−cf. **8** and **7**, respectively).

The planarity of these flavonols also appears to be important for activity: the hydrogenated form of quercetin (**6**), taxifolin (**10**, $IC_{50} = 40.1 \pm 3.6$ μM) is approximately threefold less active that quercetin, and molecular modelling Fig. 5) shows marked deviation from planarity in taxifolin relative to quercetin, and also myricetin. Moving the 3′-hydroxy group on quercetin to the 2′-position to give morin (**5**), which causes twisting of the phenyl ring

Table 1
Inhibition of RCM-κ-CN fibril formation by test compounds compared with literature values for $A\beta_{1-40}$ and $A\beta_{1-42}$

Compound	RCM-κ-casein [a]		$A\beta_{1-40}$	$A\beta_{1-42}$
	%FFi (single dose)	$IC_{50} \pm SEM$ (µM)	IC_{50} (µM)	IC_{50} (µM)
Catechins				
1	100	3.7 ± 0.3	0.18[b]	—[h]
2	0	—	2.9[c]	5.3[c]
3	7	—	2.8[c]	5.6[c]
4	17	—	—	—
Flavonols and related compounds				
5	99[i]	49.5 ± 16.3	0.24[c]	0.67[c,h]
6	84	13.6 ± 3.8	0.24[c]	0.72[c,h]
7	86[i]	33.0 ± 2.6	1.7[c]	3.2[c]
8	92	8.5 ± 0.7	0.29,[c] 0.9,[d] 0.2[e]	0.40,[c] 0.34[e]
9	68	61 ± 44	Inactive[f]	—
10	84[i]	40.1 ± 3.6	—	—[h]
11	89[i]	13.9 ± 9.7	—	—
12	0	—	Enhancer[g]	
13	90[i]	6.8 ± 1.8	—	—
14	0	—	—	—
15	35	—	—	—
Isoflavones				
16	95	190 ± 91	Moderate[f]	—[h]
17	25[i]	—	Weak[f]	—
18	0	—	—	—
19	1	—	Inactive[f]	—

[a] SEM = standard error of mean. FFi = inhibition of fibril formation. Concentrations used in single dose screen: **4**; 100 µg/mL: **1–3**, **5–7**, **9**, **10**, **13**, **15–17**; 50 µg/mL: **8**; 17 µg/mL: **14**; 12.5 µg/mL: **11**, **12**, **18**, **19**; 5 µg/mL.
[b] Ref. 14.
[c] Ref. 15.
[d] Ref. 16.
[e] Ref. 17.
[f] Ref. 18.
[g] Ref. 19.
[h] Single dose $A\beta_{1-42}$ % FFi: **1**, **5**, **6**, **10**, **16** = 100, 94, 95, 57, 43, respectively.
[i] Average of 2–4 determinations.

out of the plane of the chromen-4-one rings (Fig. 5), also reduces FFi activity (**5**, IC_{50} = 49.5 ± 16.3 µM).

The activities of the isoflavones (**16–19**) ranged from low to undetectable. The most highly hydroxylated isoflavone, and the one with the preferred hydroxylation pattern on the benzene ring of the chromen-4-one moiety, genistein (**16**), was the most active but was still much less active than the majority of flavonols tested.

Of the catechins tested, only EGCG (**1**) showed measureable FFi activity with RCM-κ-CN. This result supports the SAR associated with the flavonols; 3,4,5-trihydroxyphenyl moieties appear to have a strong positive effect on FFi activity. EGCG has two components of this type and is the most active compound examined in this study. Removal of the gallate ester and the 5′-hydroxy group to give (−)-epicatechin (**3**) led to a marked loss in detectable activity.

The set of compounds used in this study can also be used to gauge the effect of methylation and acetylation of hydroxy groups on activity: The results obtained with **14**, **18** and **19** suggest that methylation has a strong detrimental effect on FFi activity, with the clearest example coming from the activity of biochanin A (**18**) relative to its unmethylated analogue genistein (**16**). This simple change, the methylation of a single hydroxy group, led to a complete loss of detectable FFi activity. The contrast in activities of related compounds **11** and **14** is stark, and the lack of FFi activity of **14** presumably results from the combined effects of trimethylation and the presence of a substituent at the 2′-position. More intriguing is the effect of acetylation. The per-acetylation of quercetin to give **13** actually led to enhanced FFi (IC_{50} = 6.8 ± 1.8 µM), with this derivative being second only to EGCG in anti-fibrilisation activity with RCM-κ-CN.

The chalcone (**15**) was included in the set of test compounds to determine if a ring-opened form of a polyphenol would show im-

proved activity. This compound is essentially a methylated and ring-opened form of quercetin (**6**), and was found to be only weakly active. Given the apparent correlation between planarity and FFi activity, this is not surprising. As **15** is highly methylated, however, and it has already been established that methylation greatly diminishes FFi activity with RCM-κ-CN, little can be concluded about the effect of opening the chromone ring. It can only be deduced that opening the chromone ring does not compensate for the detrimental effect of methylation.

Gazit and co-workers have discussed in detail the molecular mechanisms of the anti-amyloid effects of polyphenols, based on results obtained with disease-specific amyloidogenic proteins.[14,21] They have suggested that a number of common features of polyphenols make them excellent generic inhibitors of fibril formation, including their ability to hydrogen bond to the polypeptide back bone of amyloidogenic proteins through phenolic hydroxy groups, and to form aromatic π-stacking interactions with the aromatic amino acid side chains common to the amyloidogenic core of most amyloid-forming sequences. More recently it has been suggested that the inhibition of amyloid oligomerisation by polyphenols like quercetin (**6**) may be related to the latter's aggregation properties.[22] Bulic et al. have proposed that the ordered self-assembly demonstrated by many inhibitors of fibril formation may actually be key to their activity.[23] Such assembly of inhibitors would also involve aromatic π-stacking and hydrogen bonding interactions, both between the small molecules and between the forming fibrils and the small molecule assemblies.

The results obtained in the current study suggest that RCM-κ-CN interacts with polyphenols in a similar way to other amyloidogenic proteins. In particular, a high degree of hydroxylation appears to be important for good FFi with RCM-κ-CN, with the
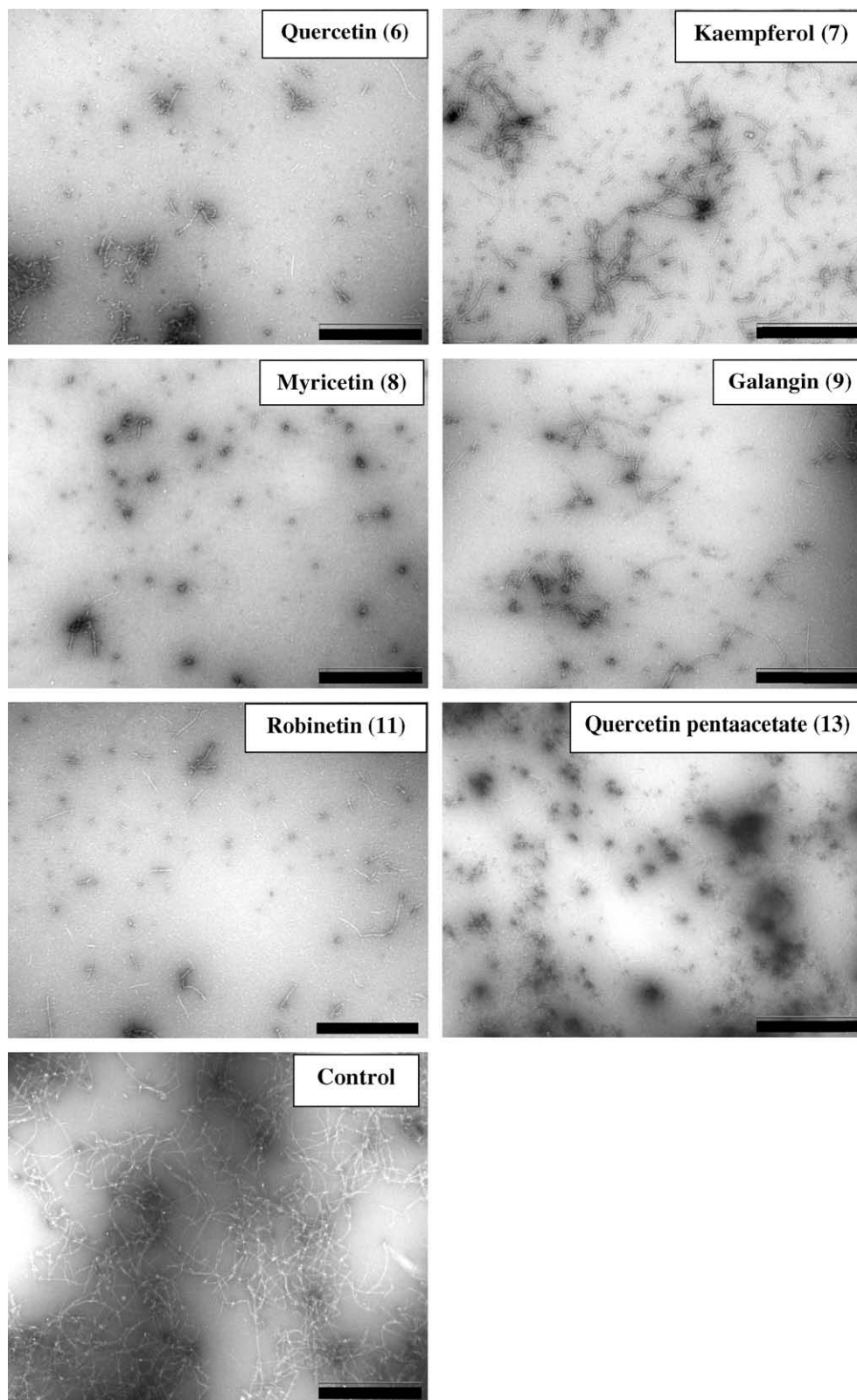
Figure 4. TEM micrographs of samples taken from completed ThT fluorescence assays. Control contained 1% DMSO but no other additive. Bars represent 1 μm.

most highly hydroxylated compounds in each of the catechin, flavonol and isoflavone classes being the most active. In support of this, methylation leads to a loss of activity. This would imply that hydrogen bonding to the native-like state of RCM-κ-CN, stabilisation of this form and limiting its fibrilisation, is not unique to the recently characterised interaction with EGCG,[10] but is a general phenomenon with polyphenols. The 3,4,5-trihydroxyphenyl moiety, in particular, appears to be highly beneficial to FFi activity.

The result obtained with quercetin pentaacetate (**13**) is surprising as this compound can only act as a hydrogen bond acceptor, not
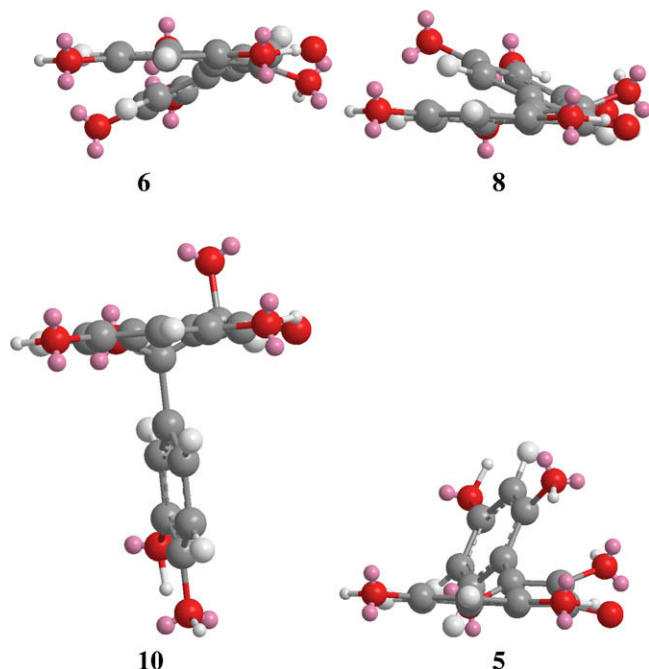
Figure 5. Energy minimised structures (MM2; Chem3D Pro 11.0) of quercetin (**6**), myricetin (**8**), (+)-taxifolin (**10**) and morin (**5**) viewed from C-6.

as a donor. One of the major challenges to the application of polyphenols for the treatment of neurodegenerative diseases is the penetration of the blood–brain-barrier (BBB). The BBB penetration of highly polar compounds like polyphenols is expected to be very limited,[24] but the result obtained with **13** suggests that acylation may be a way of improving BBB penetration of polyphenols without greatly diminishing their anti-fibrilisation activities. This result deserves further investigation.

The beneficial effect of planarity in the test compounds on FFi, as demonstrated by the activity of quercetin (**6**) relative to taxifolin (**10**) and morin (**5**), supports the notion that aromatic π-stacking interactions, as with other amyloidogenic proteins,[14,20] are important in the prevention of the fibrilisation of RCM-κ-CN, as it is expected that deviation from the plane would greatly disrupt such associations.

3.2. Comparisons with inhibition of $A\beta_{1-40}$ and $A\beta_{1-42}$ fibril formation

Literature ThT FFi results with either $A\beta_{1-40}$ or $A\beta_{1-42}$ are available for more than half of the compounds examined in this study, and these results are listed in Table 1. Where quantitative data are available (compounds **1**, **5–9**), the polyphenols studied are between 20- and 200-fold less active in the RCM-κ-CN ThT assay than in the $A\beta_{1-40}$ ThT assay, and between 10- and 74-fold less active than in the $A\beta_{1-42}$ assay. With the exception of morin (**5**) however, the order of activity is in general agreement between the assays: RCM-κ-CN; **1 > 8 > 6 > 7 > 5 > 9**, $A\beta_{1-40}$; **1 > 5 ∼ 6 > 8 > 7 > 9**, $A\beta_{1-42}$; **8 > 5 > 6 > 7**. Qualitative $A\beta_{1-40}$ data are available for another five compounds (**9**, **12**, **16**, **17** and **19**) and with the exception of galangin (**9**), the trend in activity is again in general agreement: RCM-κ-CN; **9 > 16 > 17 > 19 = 12**, $A\beta_{1-40}$; **16 > 17 > 9 = 19 > 12**. As a further comparison, we determined the %FFi with $A\beta_{1-42}$ by a sub-set of compounds; **1**, **5**, **6**, **10** and **16** (Table 1, footnote h) and the results again showed a similar trend in FFi activity to those obtained with RCM-κ-CN.

The RCM-κ-CN ThT assay described here appears to be a good predictor of anti-fibrilisation activity with Aβ, and while RCM-κ-CN IC$_{50}$ values cannot be directly correlated with those obtained with either $A\beta_{1-40}$ or $A\beta_{1-42}$, the assay's ease of operation, reproducibility and robustness under physiological conditions makes it a very useful and economic first screen for FFi activity, especially when large compound sets are being considered.

4. Conclusions

We have employed a series of flavonoids, a number of which are known to be active in Aβ fibrilisation assays, to test their ability to inhibit RCM-κ-CN fibril formation, in order to study the structure–activity relationships around the inhibition of RCM-κ-CN fibril formation and to determine if this κ-casein derivative can be used in a convenient and economic first screen for anti-fibrilisation activity of small molecules with Aβ.

A number of structural features of these flavonoids that have already been identified to be important in the inhibition of fibrilisation of disease-specific peptides like Aβ were also found to favour the inhibition of fibrilisation of RCM-κ-CN. These include a high degree of hydroxylation, which is thought to encourage the association of the test compound through hydrogen bonding with the polyamide backbone of the native-like state of RCM-κ-CN, thus stabilising this form and limiting its fibrilisation. The 3,4,5-trihydroxyphenyl substituent appears to be highly beneficial in this regard, with the most active compound tested, EGCG (**1**) possessing two such substituents. Consistent with these findings, the exchange of phenol substituents to methyl ethers has a marked negative effect on the inhibition of fibrilisation. Surprisingly the acetyled form of quercetin (**6**) led to an improvement in activity, which suggests possibilities for the promotion of BBB penetration while maintaining anti-fibrilisation activity. Co-planarity of aromatic moieties also appears to be beneficial, with structural features that cause deviation from the plane being associated with a drop in activity. This supports the notion that π-stacking interactions of the test compounds with the aromatic amino acid side chains in the amyloidogenic core of RCM-κ-CN, and/or in the self-assembly of the test compounds, also limits fibrilisation.

The polyphenols studied are between 20- and 200-fold less active in the RCM-κ-CN assay than in the $A\beta_{1-40}$ assay, and between 10- and 74-fold less active than in the $A\beta_{1-42}$ assay. This may be attributable to a number of factors which could be related to differences between RCM-κ-CN and Aβ in size, hydrophobicity, structure of the native state and the kinetics of fibril formation. With only a few exceptions, however, the order of potency of these polyphenols is in agreement for both types of protein. The RCM-κ-CN assay described here thus appears to be a good predictor of anti-fibrilisation activity with Aβ, and the assay's convenience and reproducibility makes it a valuable first screen for FFi activity, especially with large compound libraries.

5. Experimental

5.1. Materials

κ-Casein obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) was reduced and carboxymethylated as described by Shechter et al.[25] and the lyophilised product was stored at −20 °C until use. Fresh solutions of RCM-κ-CN were prepared each day and discarded after use. Compounds **1–3**, **5–10** and **16–18** are available from Sigma–Aldrich; compounds **11** and **12** are available from Indofine Chemical Co. (Somerville, NJ, USA); compound **13** is available from Maybridge via Fisher Scientific (Loughborough, Leicestershire, UK); compound **19** is available from AvaChem Scientific (San Antonio, TX, USA). (−)-Epiafzelechin (**4**) can be readily isolated from the heartwood of *afzelia* species,[26] compound **14** can

be prepared from 6,7,7′-trimethoxychromono(3′,2′,4)isocoumarin[27] by reduction with sodium borohydride[28] and compound **15** can be prepared by basic hydrolysis of pentamethyl taxifolin.[29]

5.2. Thioflavin T assay

The formation of amyloid fibrils by RCM-κ-CN was monitored using an in situ ThT binding assay.[12] A stock solution of RCM-κ-CN (1.0 mg/mL or 52 μM) was prepared in 100 mM sodium phosphate buffer (pH 7.2), centrifuged and filtered (0.22 μm) and then kept on ice before being aliquoted into the wells of a 96-well plate (see below). The test compounds were first dissolved in a small amount of DMSO and then diluted in MilliQ water such that their final concentration ranged from 10 to 200 μg/mL, depending on their solubility in DMSO. Preliminary assays showed that fibril formation by RCM-κ-CN is sensitive to the presence of DMSO and therefore the final DMSO concentration did not exceed 1%, which was not found to significantly affect fibril formation. Both the RCM-κ-CN and test compounds were then diluted twofold into black μClear 96-microwell plates (Greiner Bio-One, Stonehouse, UK) such that the final concentrations were 0.5 mg/mL (RCM-κ-CN), 5–100 μg/mL for the test compounds and the phosphate buffer concentration was 50 mM (pH 7.2) in a total volume of 200 μL. ThT from a stock solution made up in phosphate buffer (50 mM, pH 7.2) was added to give a final concentration of 10 μM. The plates were sealed (thin seal self-adhesive plate covers, Excel Scientific, Chicago, IL, USA) to prevent evaporation and incubated at 37 °C without shaking. The time between when the lyophilised RCM-κ-CN was first dissolved in buffer and when the stock solution was diluted into the plate and the assay commenced was approximately 1 h. The fluorescence intensity of each well was recorded every 5 min for 1000 min using a Fluostar Optima plate reader (BMG Labtechnologies, Melbourne, Australia) with a 440/490 nm excitation/emission filter. All samples were prepared in duplicate. The percent inhibition of fibril formation (FFi%) was calculated from the change in ThT fluorescence in the absence and presence of the compounds as follows: %FFi = $100 \times (\Delta Fc - \Delta Fs)/\Delta Fc$, where ΔFc and ΔFs represent the change in ThT fluorescence in the absence (control) and the presence of test compound, respectively. For those compounds that gave a high %FFi (i.e., usually 80% or higher), a dose–response study was performed over four different concentrations of test compound and an IC_{50} value was determined using GraphPad PRISM 5.01 (GraphPad Software, San Diego, CA, USA). EGCG (**1**) was included as a positive control in each batch of test compounds. The four individual IC_{50} values thus determined for this compound were in close agreement and the value shown in Table 1 is an average of these four numbers.

5.3. Transmission electron microscopy

Samples for TEM were prepared by taking 5 μL of each sample directly from the ThT assay and applying it to a carbon-coated 400-mesh nickel TEM grid (ProSciTech, Australia). The grid was then washed with 0.22 μm filtered MilliQ water (3 × 10 μL) and negatively stained with uranyl acetate solution (5 μL, 2% w/v, in MilliQ water). Grids were dried with clean filter paper between each of these steps. After being left to air dry, the samples were viewed at 19,000× magnification using a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands).

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