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Original article

Synthesis and testing of peptides for anti-prion activity

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Abstract

Creutzfeldt–Jakob disease (CJD) is one of the fatal transmissible spongiform encephalopathies for which there is no known cure. The disease is associated with an abnormally folded prion protein, PrP-res, which is thought to form due to interaction between normal prion PrP^C and PrP-res. Small peptides were designed to prevent this interaction. A structure–activity relationship is described for a series of peptides which were synthesised and tested for their activity against two prion disease model assays, an *in vitro* cellular assay and an *in vitro* anti-aggregation polymerisation assay. A number of peptides were found to be active at levels of 100 μM. New libraries were synthesised in order to concentrate on discovering new, shorter peptides which could be leads for developing peptidomimetics.

Keywords: Prion; Transmissible spongiform encephalopathy; Peptide

1. Introduction

The transmissible spongiform encephalopathies (TSEs) are a diverse range of diseases affecting humans and animals [1]. They cause neurological disorders associated with the aggregation of an isoform of a host-encoded protein, termed the prion protein (PrP) [2], and are fatal with no known cure as yet.

The infectious form of PrP (denoted as PrP-res) has the same amino acid sequence as the normal form (PrP^C) [3]. The difference between PrP^C and infectious PrP-res lies in their conformations; PrP^C is predominantly α-helical with little β-sheet content, whilst PrP-res has a higher level of β-sheet than α-helical structure. The β-sheets have a propensity to aggregate and form insoluble amyloid plaques [4]. When PrP-res comes into contact with normal PrP^C, it is thought to somehow cause the normal conformation to change into the disease causing conformation. This event starts a cascade in which newly converted infectious prions change the shape of normal PrP^C molecules. This cascade is thought to occur via internalisation from the cell surface.

The treatment of transmissible spongiform encephalopathies (TSEs) requires the development of new therapeutic agents [5,6]. A number of lead compounds have already been reported to ''cure'' persistently scrapie-infected cells and to have some *in vivo* effect in rodent models of infection [7]. These include the sulfated polysaccharides such as pentosan polysulfate [8], quinacrine [9], diazo dyes such as Congo Red [10–14], porphyrins and phthalocyanines [15], amphotericin B [16,17], polyamines [18] and anthracyclines such as IDX [19]. Many compounds are not drug candidates themselves because of insufficient brain barrier permeation and/or poor *in vivo* activity. The precise mechanism of action is not known for most of these compounds.

The aim of the work described in this paper was to investigate if small peptides could be developed which show anti-TSE activity in cellular and cell-free models; these could potentially be leads for a drug discovery programme. During the conversion of PrP^C to PrP-res it is believed that there is a protein–protein interaction between PrP^C and PrP-res. Peptides based on PrP should interfere with this interaction. It is important to

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select relatively small peptides, as we ideally would like to eventually produce a small molecule inhibitor of the process, potentially one that can cross the blood–brain barrier. Peptides themselves generally make poor drug candidates, due to problems with bioavailability and enzymatic degradation; therefore we envisaged converting the peptide leads into peptoids or peptidomimetics. There are a number of successful methodologies for the conversion of peptide leads into drug candidates using peptidomimetics [20].

1.1. Strategy

Our strategy was based on the hypothesis that residues on the surface of the normal prion protein will interact with the abnormal protein. This interaction is likely to involve residues including the region 100–141. Chabry et al. made a series of peptides spanning the length of prion protein and found that those involving this region inhibited a cell-free conversion assay of PrP-sens to PrP-res [21]. Peptides 109–141 and 106–128 blocked PrP-res formation in cell-free conditions. In a second paper, they reported that peptides representing the region 119–128 did not show any inhibition in the cell-free conversion assays but those of the region 119–136 had a strong inhibitory effect in both cell-free and living cell conditions [22]. Peptides of this sequence were able to inhibit PrP-res formation in persistently scrapie-infected neuroblastoma cells (IC_{50}, ~11 μM). Peptide 129–136 is thought to be important in PrP–PrP interaction *in vitro* and *in vivo* and it also influences the inhibition of PrP conversion [22].

There are three possible mechanisms of action: (i) the peptides might bind to PrP^C either stabilising it or preventing its interaction in the conversion to PrP-res; (ii) the peptides might bind to PrP-res preventing it from acting as a ''seed'' and propagating the conversion reaction. Peptides may also achieve this by mimicking part of the surface of PrP [23]; (iii) furthermore it has been speculated that the process of conversion of PrP-sens to PrP-res requires the presence of an intermediary chaperone (protein X) [24]. A possible third mode of action of these peptides is the binding to this chaperone molecule, preventing its ability to participate in the binding of either PrP-sens or PrP-res.

Based on previous work published by Chabry et al. [21,22], we carried out a study of the prion protein sequence 109–141 (Syrian Hamster numbering) in humans, bovine and hamster to allow design of new peptide libraries to discover potential leads for further development. Peptides found to have activity were then subject to further structure–activity relationship studies (proline scans, residue deletion) to identify the relative stereochemical locations of the most important amino acid chains involved in the conversion process.

2. Molecular modelling

A molecular modelling and sequence alignment study was carried out in order to compare the structures of the normal (PrP^C) prion protein from human, cattle and hamster. The sequences of the bovine, ovine, human and hamster prion proteins were aligned using Clustal (Fig. 1) [25]. They have

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CLUSTAL W (1.83) multiple sequence alignment


Bovine          MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGW 60
Ovine           MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGW 60
Human           --MANLGCWMLVLFVATWSDLGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPPQGGGGW 57
Hamster         --MANLSYWLLALFVATWTDVGLCKKRPKPGG-WNTGGSRYPGQGSPGGNRYPPQGGGTW 57
                   :::. *:*.**** *:*:*********** ************************ *

Bovine          GQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-THGQWNKPSKPKTN 119
Ovine           GQPHGGGWGQPHGGGWGQPHGG--------GWGQPHGGGGWGQGG-SHSQWNKPSKPKTN 111
Human           GQPHGGGWGQPHGGGWGQPHGG--------GWGQPHGGG-WGQGGGTHSQWNKPSKPKTN 108
Hamster         GQPHGGGWGQPHGGGWGQPHGG--------GWGQPHGGG-WGQGGGTHNQWNKPNKPKTS 108
                ********************         ********* ***** :*.***** ****.

Bovine          MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYYRENMHRYPNQVYYRPVDQ 179
Ovine           TKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQ 171
Human           MKHMAGAAAAGAVVGGLGGYMLGSAMSRPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDE 168
Hamster         MKHMAGAAAAGAVVGGLGGYMLGSAMSRPMLHFGNDWEDRYYRENMNRYPNQVYYRPVDQ 168
                 **:************************:::***.*:********* **********:*:

Bovine          YSNQNNFVHDCVNITVKEHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQ- 238
Ovine           YSNQNNFVHDCVNITVKQHTVTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQ- 230
Human           YSNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQ- 227
Hamster         YNNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKMMERVVEQMCVTQYQKESQAYYDG 228
                *.************:*:****************:*:*********:***::******:

Bovine          RGASVILFSSPPVILLISFLIFLIVG 264
Ovine           RGASVILFSSPPVILLISFLIFLIVG 256
Human           RGSSMVLFSSPPVILLISFLIFLIVG 253
Hamster         RRSSAVLFSSPPVILLISFLIFLIVG 254
                * :* :********************
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Fig. 1. Sequence alignment of the bovine, ovine, human and hamster prion protein sequences.

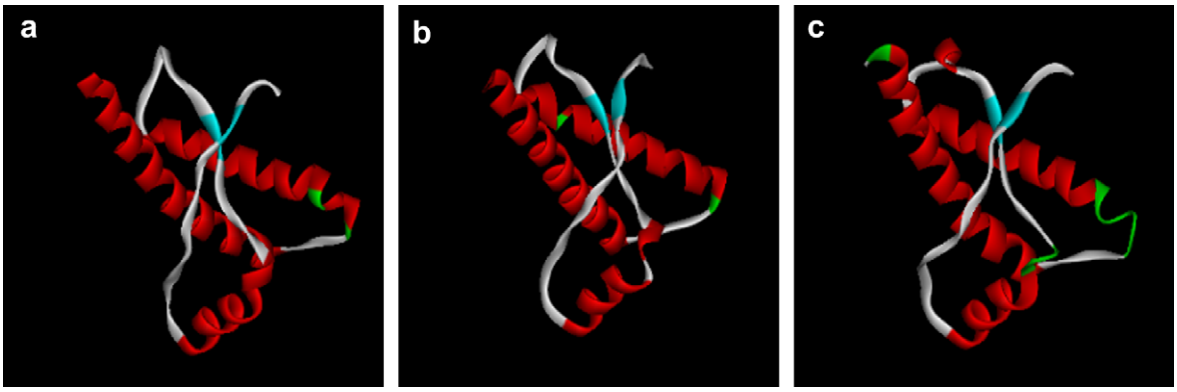


Fig. 2. Structures of the (a) human (1QLX); (b) bovine (1DX0); (c) hamster prion proteins (1B10).

a very high sequence identity and show a very similar structure (as would be expected from their high sequence similarity) containing three α-helices and a small β-sheet (Fig. 2).

The sequence 109–141 seems to play an essential role in the conversion process but the rest of the protein does not. This region is located on the outer domain of the prion protein for the three species and contains the β-sheet of the normal prion protein. This might represent the main point of contact of the abnormal prion protein with the normal prion protein during disease propagation (Fig. 3).

3. Chemistry

Peptides were prepared using solid phase Fmoc amino acid coupling chemistry (Scheme 1). This was carried out using rink amide MBHA resin, which upon final cleavage gave a C-terminal primary amide. This should improve the enzymatic stability of the peptides.

4. Biological assays

Two assays were used for assessment of the compounds: a cellular assay using a persistently infected cell line and a cell-free assay. The cellular assay used the scrapie mouse brain (SMB) cell line assay. The SMB cells are persistently scrapie-infected mouse cells cloned from a scrapie-infected mouse brain, but of non-neuronal origin. These cells are highly phagocytic and may mimic cells involved in the initial uptake and replication of the agent in peripheral infection. In addition these cells show stable persistent scrapie-infection over many passages, suggesting that they are suitable for drug screening. We have never observed the SMB cells to loose their infection or ''self-cure''. Indeed we have successfully used this cell line to screen many derivatives of Congo Red [10,14]. The peptides were screened at 100 μM to see the effect on PrP-res levels compared to control (untreated) cells.

In order to try and understand further the mode of action of these compounds, investigations were carried out to see if these compounds could inhibit polymerisation of recombinant PrP (rPrP) by PrP-res in a cell-free assay. The seed for polymerisation was recombinant mouse PrP which was refolded and aggregated using manganese ions, or PrP-res extracted from SMB cells. This was then used to seed aggregation of recombinant mouse PrP (which was refolded in the absence of metal ions). The aggregation was monitored by looking at scattering of UV light. The effect of compounds on inhibiting

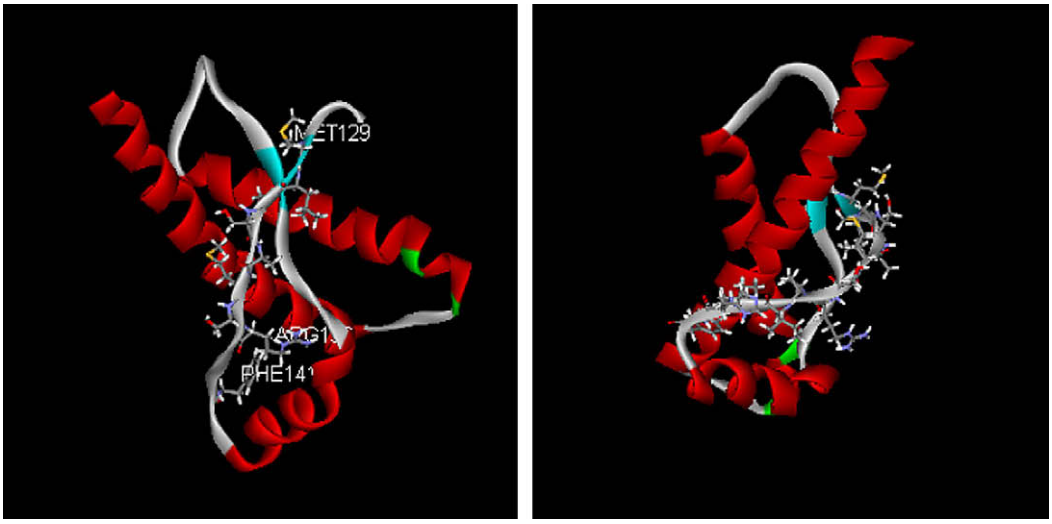
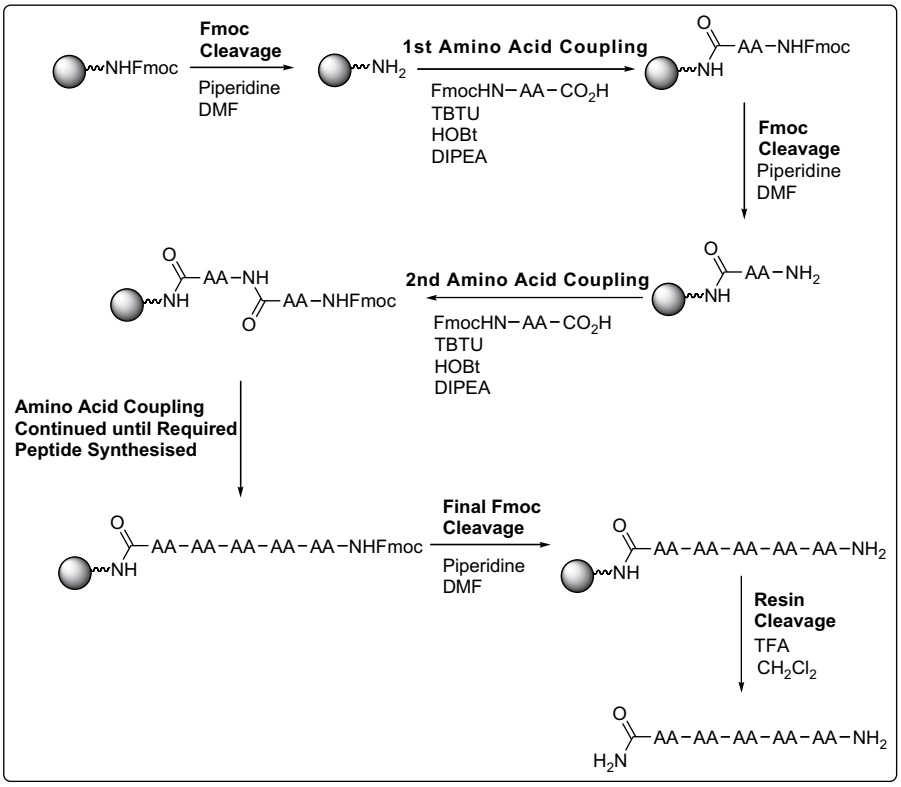


Fig. 3. Solid ribbon representation using PDB files and Viewer Lite®: human prion protein, with the sequence 129–141 as stick representation. Two different perspectives are shown.



Scheme 1. Synthesis of peptides.

aggregation was determined. Some of the compounds caused precipitation prior to addition of the seed. This means that interpretation of the data related to these compounds is unreliable (where this is the case it is marked in the tables).

5. Results

The first part of our study concentrated on the hydrophobic sequence 112–120 (Hamster numbering), which is known to be amyloidogenic (Fig. 4). As the main component of this fragment is alanine, a proline scan was performed to identify the most important amino acids involved in the conversion process (compounds **2**–**8**). Prolines are known to be β-sheet breakers [26]. By replacing each residue in turn with a proline, it should be possible to determine which are important for interaction with the molecular target. Furthermore replacement of residues by proline may add β-sheet breaking functionality to the peptide. Later on, the studied sequences were elongated and/or shortened once the biological results for the first batch of peptides were known.

Region 112-120: Synthesis of nona-peptides (highly conserved pattern) followed with a proline scan.

Human: MAGAAAAGA

Bovine: VAGAAAAGA

Hamster: MAGAAAAGA

Fig. 4. First planned sequence modification.

The valine at position 112 was replaced by an alanine and a proline scan carried out (compounds **9**–**16**). Finally peptides with a methionine at position 112 were made, together with the corresponding proline scans (compounds **17**–**23**). Full data for all compounds is presented in Supplementary data. Data for compounds showing activity is presented here (Table 1).

From the cell-free conversion assay, one peptide **14** (sequence AAGAAAPGA–NH_2) stood out particularly as it lowered the levels of PrP-res to 27% of the control levels. It did not have such a significant effect on the cellular assay. Peptide **2** also showed weak activity in the cell-free polymerisation assay. Peptides **9** and **11** showed some activity in the cellular assay. None of the other peptides showed significant activity (see Supplementary data).

Table 1
Series 1 – the original sequences (compounds **1**, **9**, **17**) and significant data from the proline scan

Compound	Structure	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
1	VAGAAAAGA–NH_2	93	84
2	PAGAAAAGA–NH_2	100	71
9	AAGAAAAGA–NH_2	56	76
11	AAPAAAAGA–NH_2	66	103
14	AAGAAAPGA–NH_2	81	27
17	MAGAAAAGA–NH_2	89	104

[a] Percentage of PrP-res remaining compared to control levels (untreated cells).

[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

Table 2
Series 2 – extended sequences of active peptides – one amino acid extension

Compound	Structure	Modification	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
24	HAAGAAAAGA–NH_2	H on N-terminal of **9**	39	59
25	HPAGAAAAGA–NH_2	H on N-terminal of **2**	23	11
26	HAAPAAAAGA–NH_2	H on N-terminal of **11**	151	93
27	HAAGAAAPGA–NH_2	H on N-terminal of **14**	183	28
28	AAGAAAAGAV–NH_2	V on C-terminal of **9**	133	57
29	PAGAAAAGAV–NH_2	V on C-terminal of **2**	112	77
30	AAPAAAAGAV–NH_2	V on C-terminal of **11**	148	28
31	AAGAAAPGAV–NH_2	V on C-terminal of **14**	129	54

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

Table 3
Series 3 – extended sequences – two amino acid extension

Compound	Structure	Modifications	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
32	HAAGAAAAGAV–NH_2	H on N-, V on C–terminal of **9**	74	34
33	HPAGAAAAGAV–NH_2	H on N-, V on C-terminal of **2**	135	74
34	HAAPAAAAGAV–NH_2	H on N-, V on C–terminal of **11**	225	38
35	HAAGAAAPGAV–NH_2	H on N-, V on C–terminal of **14**	32	64
36	KHAAGAAAAGA–NH_2	K, H on N-terminal of **9**	59	102
37	KHPAGAAAAGA–NH_2	K, H on N-terminal of **2**	83	19
38	KHAAPAAAAGA–NH_2	K, H on N-terminal of **11**	70	73
39	KHAAGAAAPGA–NH_2	K, H on N-terminal of **14**	44	52
40	AAGAAAAGAVV–NH_2	V, V on C-terminal of **9**	34	17
41	PAGAAAAGAVV–NH_2	V, V on C-terminal of **2**	132	28
42	AAPAAAAGAVV–NH_2	V, V on C-terminal of **11**	14	22
43	AAGAAAPGAVV–NH_2	V, V on C-terminal of **14**	5	52

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

It was decided to make some extended and shortened sequences of these peptides to see if we could increase the effect in the cellular conversion assay by increasing the molecular recognition of the peptide within the normal/abnormal prion protein (series 2, Table 2).

5.1. Extended sequences

A number of these compounds showed significant inhibition in the cell-free conversion assay – compounds **24**, **25**, **27**, **28**, **30** and **31**. Interestingly **24** and **25** also showed some inhibition in the cellular model of disease. Compounds **24**, **25** and **27** are N-terminal extensions by a His, whilst **28–31** are C-terminal extensions with a valine. Both extensions appear to increase activity in the polymerisation assay, but not in the cellular assay, with the exception of **24** and **25**. The reason for the difference in activity against the cellular and polymerisation assays may be due to a lack of enzymatic stability of the peptides in the cellular assay. Alternatively there may be a mechanism of action other than inhibition of polymerisation for compounds active in the cellular assay.

These results lead us to further investigate the effect of extension (series 3, Table 3). Compounds **32–35** contain both histidine at the N-terminal end and valine at the C-terminal end. Compounds **36–39** contain a two-residue extension (Lys–His) at the N-terminal end, whilst compounds **40–43** contain a two-residue extension (Val–Val) at the C-terminal end.

Almost all of these additions gave reasonable efficacy in the polymerisation assay. Compounds **35**, **39**, **40**, **42** and **43**

Table 4
Series 4 – extended sequences – four amino acid extension

Compound	Structure	Modifications	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
44	KHAAGAAAAGAVV–NH_2	KH, VV on N, C-terminal of **9**	93	112
45	KHPAGAAAAGAVV–NH_2	KH, VV on N, C-terminal of **2**	68	73
46	KHAAPAAAAGAVV–NH_2	KH, VV on N, C-terminal of **11**	68	44
47	KHAAGAAAPGAVV–NH_2	KH, VV on N, C-terminal of **14**	7	86

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

Table 5
The percentage of PrP-res formation compared to control in the cell-free polymerisation assay: comparison of chain extensions

Original peptide	**9**	**2**	**11**	**14**
Original peptide	76	71	103	27
N-term His	59	11	93	28
N-term Lys–His	102	19	73	52
C-term Val	57	77	28	54
C-term Val–Val	17	28	22	52
N-term His; C-term Val	34	74	38	64
N-term Lys–His; C-term Val–Val	112	73	44	86

also gave at least 50% reduction in levels of PrP-res in the cellular assay. The Val–Val extension at the C-terminal end appeared to have the most affect on the cellular assays.

The addition of a four amino acid extension (i.e. two amino acids to the C-terminal and two to the N-terminal) (series 4, Table 4) provided two new significantly active compounds: compound **47** which reduced the control levels to 7% of the original in the cellular assay, and **46** which reduced polymerisation control levels to 44% of original levels.

It is interesting to do a comparison of activities in the polymerisation assay across the series (Table 5). Unfortunately there is no complete consensus, but the following points may be made.

- The N-terminal His increased activity.
- The double extension at both ends was detrimental.
- With the exception of **14**, a C-terminal Val–Val extension improved activity.

This lack of total consensus is perhaps not surprising, as each original peptide will have a slightly different conformation and hence additional residues will have differing effects on the activity.

5.2. Residue truncations

We were also interested to see the effect of removing residues from the peptides; this would allow us to reduce the size of peptides. For three peptides (**9**, **11**, **14**) this led to a reduction in activity in the cellular assay, but an increase in activity in the polymerisation assay. The converse was observed for **2** (Table 6).

Deletion of two amino acid residues from the C-terminal end had the same effect in the cellular assays. The peptides were too insoluble for the cell-free polymerisation assay, with the exception of **54**, which reduced PrP-res formation to just 2% of control levels (Table 7).

Table 6
Series 5 – shortened sequences of active peptides – one amino acid truncation

Compound	Structure	Modifications	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
48	AAGAAAAG–NH_2	A deleted on C-terminal of **9**	108 (56)[c]	23 (76)
49	PAGAAAAG–NH_2	A deleted on C-terminal of **2**	10 (100)	130 (71)
50	AAPAAAAG–NH_2	A deleted on C-terminal of **11**	128 (66)	46 (103)
51	AAGAAAPG–NH_2	A deleted on C-terminal of **14**	108 (81)	2 (27)

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).
[c] Data in parentheses refer to data of the original (untruncated) peptide.

Table 7
Series 6 – shortened sequences – two amino acid truncation

Compound	Structure	Modifications	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
52	AAGAAAA–NH_2	GA deleted on C-terminal of **9**	65 (56)[c]	pia
53	PAGAAAA–NH_2	GA deleted on C-terminal of **2**	71 (100)	pia
54	AAPAAAA–NH_2	GA deleted on C-terminal of **11**	195 (66)	2 (71)
55	AAGAAAP–NH_2	GA deleted on C-terminal of **14**	206 (81)	pia

pia refers to precipitated in assay.
[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).
[c] Data in parentheses refer to data of the original (untruncated) peptide.

5.3. A proline scan on compound *14*

A second proline scan on **14** provided more information for our study; **57** and **58** were particularly active in the cellular assay, whilst peptide **63** was active in the polymerisation assay (Table 8). (Data of all compounds are provided in Supplementary data.)

5.4. The region 129–136

Preliminary work was also conducted on the region 129–136, which has also been shown to influence the inhibition of PrP conversion. The same sequence is found here in the human, bovine and hamster protein: MLGSAMSR.

A library of octapeptides were synthesised and a proline scan performed. Promisingly the two proline analogues that

Table 8
Series 7 – second proline scan of **14**

Compound	Structure	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
14	AAGAAAPGA–NH_2	81	27
56	PAGAAAPGA–NH_2	79	56
57	APGAAAPGA–NH_2	7	89
58	AAPAAAPGA–NH_2	33	85
63	AAGAAAPGP–NH_2	88	27

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

Table 9
Series 8 – the initial second sequence modification (proline scan)

Compound	Structure	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
64	PLGSAMSR–NH_2	45	11
65	MLGSAPSR–NH_2	61	1

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

were prepared had good activity in both the cellular and cell-free polymerisation assays (Table 9).

6. Discussion

To facilitate analysis of the data, peptides were analysed in order of activity. Table 10 shows the 10 most active peptides at 100 μM in the cellular assay.

Some peptides were also investigated in our cellular assay at 10 μM concentration. The data (Table 11) show that the IC_{50} values for these peptides lie above 10 μM. Indeed at slightly lower concentrations, some of these peptides appear to increase the amount of PrP-res. We observed a similar effect with Congo Red [13].

Two methods were used for the polymerisation assay: the seed used for the assay was either a recombinant PrP seed which had been converted to the protease resistant form or PrP-res extracted from SMB cells. The most potent compounds are shown in Table 12.

Analysis of these data indicated a recurring motif: GAAAPG which occurs in many active motifs in both the cellular and cell-free assays. Therefore we decided to investigate this series further: (i) to carry out a second proline scan on this sequence (Table 13), to see if we could identify residues which may be important for activity; (ii) to reduce the peptide size to 4 or 3 residues, also carrying out a proline scan with these.

Series 10 (Table 13) is the proline scan of GAAAPG. When these compounds were tested, one peptide, **68** (GAAPPG–NH_2), reduced levels of PrP-res to 5% of control levels at 100 μM in the cellular assay, but also unusually seemed to stimulate aggregation in the polymerisation assay. Compounds

Table 10
The most active compounds in the cellular assay at 100 μM

Compound	Structure	Cellular data[a] (100 μM)
43	AAGAAAPGAVV–NH_2	5
57	APGAAAPGA–NH_2	7
47	KHAAGAAAPGAVV–NH_2	7
49	PAGAAAAG–NH_2	10
42	AAPAAAAGAVV–NH_2	14
25	HPAGAAAAGA–NH_2	23
35	HAAGAAAPGAV–NH_2	32
58	AAPAAAPGA–NH_2	33
40	AAGAAAAGAVV–NH_2	34
24	HAAGAAAAGA–NH_2	39

[a] Percentage of PrP-res remaining compared to control levels.

Table 11
Cellular assays repeated for the most active compounds at 10 μM

Compound	Structure	Cellular data (10 μM)
43	AAGAAAPGAVV–NH_2	73
47	KHAAGAAAPGAVV–NH_2	108
42	AAPAAAAGAVV–NH_2	131
49	PAGAAAAG–NH_2	158
40	AAGAAAAGAVV–NH_2	200

67 and **69** also appeared to have some activity. However, in general this motif on its own was not effective. This suggests that whilst the GAAAPG motif is commonly found in active peptides, it is not of itself sufficient for activity, and that simple changes do not in general increase activity.

Similarly amongst the tetrapeptides, based on GAAAPG and proline scans thereof (series 11, Table 13), there was little activity. Peptide **81** showed some efficacy in the cellular model. However, surprisingly some of the tripeptides (series 12, Table 13) showed activity: **84**, **85**, **90** and **93** in the cellular assay and **92** in the polymerisation assay.

It is interesting to note that some peptides cause an increase in the level of PrP-res detected in the cellular assay or increased the extent of PrP aggregation in the cell-free polymerisation assay. The explanation for this is uncertain. In the cell assay this could imply that the peptides stabilise the PrP-res produced by the cells, decreasing any amount of degradation that occurs or stimulate the rate of protein conversion to PrP-res. In the polymerisation assay the increase could be due to stimulation of PrP aggregation or aggregation of the peptide itself.

7. Conclusions

A number of peptides that we have prepared inhibited PrP-res formation either in the cellular assay or the cell-free

Table 12
The most potent compounds in the polymerisation assay at 100 μM

Compound	Structure	Polymerisation data[a] (100 μM) (using SMB cell lysate)
51[b]	AAGAAAPG–NH_2	2
54[b]	AAPAAAA–NH_2	2
25[b]	HPAGAAAAGA–NH_2	11
40[b]	AAGAAAAGAVV–NH_2	17
37[c]	KHPAGAAAAGA–NH_2	19
42[b]	AAPAAAAGAVV–NH_2	22
48[b]	AAGAAAAG–NH_2	23
14[c]	AAGAAAPGA–NH_2	27
63[c]	AAGAAAPGP–NH_2	27
27[c]	HAAGAAAPGA–NH_2	28
30[c]	AAPAAAAGAV–NH_2	28
41[b]	PAGAAAAGAVV–NH_2	28
46[b]	KHAAPAAAAGAVV–NH_2	44
50[b]	AAPAAAAG–NH_2	46
43[b]	AAGAAAPGAVV–NH_2	52

[a] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).
[b] Seeded with SMB PrP-res extract.
[c] Seeded with recombinant PrP treated with manganese ions.

Table 13
Investigation of the sequence GAAAPG (selected compounds)

Compound	Structure	Cellular data[a] (100 μM)	Polymerisation data[b] (100 μM)
Series 10			
66	GAAAPG–NH_2 original lead	124	111
67	GAAAPP–NH_2	30	148
68	GAAPPG–NH_2	5	240
69	GAPAPG–NH_2	52	85
70	GPAAPG–NH_2	261	48
Series 11			
74	AAPG–NH_2	100	88
81	GAPA–NH_2	47	266
Series 12			
84	GPP–NH_2	25	81
85	PPA–NH_2	10	125
88	APA–NH_2	45	148
89	PAA–NH_2	51	88
90	PPG–NH_2	48	96
92	PAP–NH_2	156	59
93	GAP–NH_2	32	111

[a] Percentage of PrP-res remaining compared to control levels.
[b] Percentage of PrP-res formed from rPrP (recombinant PrP) compared to control levels (untreated assays).

polymerisation assay. We have demonstrated that relatively small peptides (3–13-mers) can have activity in either the cellular or cell-free assay. Peptides of particular interest are shown in Table 14.

Whilst the peptides themselves are not suitable drug candidates, a peptidomimetic approach using these leads may give rise to compounds with drug-like properties. Indeed there are a number of examples where drugs or drug-like compounds have been derived by modification of peptides to make peptoids. Thus the first generation HIV-protease inhibitors were designed starting from peptides. The amide bond that is cleaved was replaced by bio-isosteres of the amide bonds, and then finally other residues in the peptides were systematically optimised to give saquinavir [27,28]. Pritchard et al. [20] report a method for generation of peptoids from peptides, in which the most important amino acid side chains in a peptide are identified and substituted on an appropriate scaffold or template. Methylation at either the α-carbon or on the amide nitrogen reduces the conformational flexibility of the peptides and can be used to investigate the stereochemical requirements of the side chains for activity. Knor et al. [29] report strategies to give a peptidomimetic of an octapeptide ligand that is used for purification of Factor VIII. They used strategies based on scans with alanine, scans with D-amino acids, systematic replacement of residues with other amino acids or mimics of amino acids, truncations, and modifications of the peptide bonds.

Table 14
Peptides of particular interest

Compound	Structure	Cellular (100 μM)	Polymerisation
43	AAGAAAPGAVV–NH_2	5	
68	GAAPPG–NH_2	5	
57	APGAAPGA–NH_2	7	
47	KHAAGAAAPGAVV–NH_2	7	
49	PAGAAAAG–NH_2	10	
42	AAPAAAAGAVV–NH_2	14	
65	MLGSAPSR–NH_2		1
51	AAGAAPG–NH_2		2
54	AAPAAAA–NH_2		2
25	HPAGAAAAGA–NH_2		11
64	PLGSAMSR–NH_2		11

In the research described here, in general there was not a significant overlap between compounds that were active in the cellular assay and those that were active in the cell-free polymerisation assay. There could be several explanations. Firstly compounds that are active in the cellular assay, but not the polymerisation assay, are not working by inhibition of polymerisation. Secondly peptides that are active in the cell-free polymerisation assay but not in the cellular assay may not show activity in the cellular assay due to either degradation in the cell culture medium or lack of uptake into cells. This would of course be an issue to be addressed in any subsequent optimisation programme.

From analysis of the most active peptides, it appears that many peptides containing the motif GAAAPG appear to have activity in either the cell assay and or the polymerisation assay, although this motif by itself is not active. However, this is a motif worthy of further exploration. When derivatives or smaller or fragments of GAAAPG were made, they did show some activity in cellular or cell-free assays. Peptides active in the cellular assay contained a PP motif. This may well be due to the β-sheet breaking ability of the prolines. These smaller peptides are of particular interest, as there is significant potential to optimise these leads, whilst retaining a small size.

The preliminary work on the sequence 126–136 revealed two peptides that were active in both the cellular and cell-free polymerisation assays. This region is worthy of further investigation.

8. Experimental

8.1. Solid phase synthesis of peptides

8.1.1. Resin preparation

Fmoc-protected rink amide MBHA resin was swelled in DMF for 20 min, and the Fmoc group removed using 20% piperidine in DMF, and shaked for 30 min. The resin was washed with DMF and dichloromethane.

8.1.2. Amino acid coupling

The resin was shaken in DMF with TBTU (4 equiv.), HOBt (4 equiv.), DIPEA (4 equiv.) and Fmoc-protected amino acid (4 equiv.) for 1 h. After washing with DMF and dichloromethane, a few beads were assayed using ninhydrin (as above) to ensure there were no unreacted amino groups.

8.1.3. Fmoc deprotection

The resin was shaken with 20% piperidine in DMF for 30 min, followed by washing with DMF and dichloromethane.

Finally the resin could be shrunk using methanol and thoroughly dried on the vacuum pump to remove any solvent traces.

8.1.4. Cleavage from the resin

The cleavage of the peptide from the resin was carried out by shaking the resin for 20 min in TFA (95%) and water/triisopropylsilane (2.5%/2.5%) as scavengers. After removal of TFA and scavengers using nitrogen gas, the resultant residue was treated with cold diethyl ether, which led to the peptide precipitating as a white solid. The peptide was washed with more cold diethyl ether (twice), then dissolved in water and lyophilised to give the peptides as white solids.

8.1.5. Ninhydrin assay

After each stage of the process, a ninhydrin assay was carried out to detect free NH_2 groups. A few beads were heated to 100 °C for 2 min in a solution of 5% ninhydrin in ethanol.

8.1.6. HPLC

Analytical HPLC was carried out using an isocratic eluent water/acetonitrile (70:30) and a flow rate of 1 mL/min, with a Phenomenex Sphereclone 5 μ ODS-2 C18 reverse phase (240 × 4.6 mm) column.

The HPLC system used was a Beckman Coulter System Gold pump module 126 and diode array detector module 168 with 32karat software version 5.0.

Analytical data for peptides are presented in Supplementary data.

8.2. Cell culture assays

SMB cells were grown in tissue culture treated flasks in 199 medium (Gibco) supplemented with 10% (v/v) foetal calf serum, 5% (v/v) newborn calf serum (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2.

8.2.1. Peptide treatment

Peptides were resuspended in DMSO at a stock concentration of 10 mM. To assess the effects of a peptide, SMB cells were plated at 50% confluency per well in 6-well plates. Cells were left for 24 h to allow for attachment. Medium was then replaced with fresh medium containing the appropriate dilution of the 10 mM drug stock. Four days after the addition of peptide the medium was removed and the cellular protein extracted.

8.2.2. Protein extraction

Cells were lysed in PBS containing 1% Triton X-100, 1% Igepal CA-630 for 20 min at 37 °C. Cell lysates were either placed on ice or treated with 80 μg/mL proteinase K for 1 h at 37 °C. Proteins were concentrated from the total cell lysate by methanol precipitation and the protein pellet resuspended and denatured by boiling for 5 min in 8 M urea.

8.2.3. Western blotting

Samples were electrophoresed on a 12% acrylamide gel and transferred electrophoretically to PVDF membrane (Immobilon-P, Millipore). PrP was detected using the primary antibody DR1 as previously described [30] and a HRP conjugated secondary antibody (Dako). Specific protein bands were visualised using ECL Plus chemiluminescent reagent (Amersham Pharmacia Biotech) followed by autoradiography. Autoradiographs were analysed using Scion Image densitometric software (Scion Corporation).

8.3. In vitro aggregation assay

All measurements were performed on a Cary 100Bio UV–visible spectrophotometer at 325 nm using a quartz cuvette of 5 mm path length. Substrate recombinant mouse PrP (rPrP) was refolded in the absence of metal ions and the seed for aggregation was aged manganese refolded recombinant mouse PrP (MnPrP) prepared as previously described [31,32]. Briefly a seed of MnPrP induces immediate aggregation of substrate rPrP, observed as an increase in solution turbidity. The resultant scattering of UV light at 325 nm results in an increased absorbance measurement. The abilities of the potential anti-TSE compounds to prevent this turbidity increase were measured and the results expressed as a percentage of the turbidity observed with a DMSO control. rPrP of 50 μg and 100 μM peptide were preincubated in 500 μl H_2O, pH 6.5 for 30 min to provide a background zero for the measurement. MnPrP seed of 10 μg from a 400 μg/mL stock was added to the peptide/rPrP mixture and an initial reading obtained immediately. A second reading was measured after 5 min and the increase in absorbance over 5 min recorded. Time in the spectrophotometer beam was minimised due to the sensitivity to UV light of some Congo Red derivatives.

For some experiments MnPrP was not used but SMB PrP-res extract was used to seed the reaction. Protein extracts were made from SMB cells as described above, PK treated and precipitated with methanol. The pellets were dried and resuspended in water. After quantitation of the protein content, 0.1 μg of PrP-res was used to seed polymerisation in place of MnPrP in the assay described above.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2008.01.041.

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