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(54) **Title:** PRION PROTEIN DERIVED CELL PENETRATING PEPTIDES AND THEIR USES

--MANLG CWMLVLFVATWSD LGLC KKRPKP	human
--MANLGYWLLALFVTMWTDVGLC KKRPKP	mouse
MVKSHIGSWILVLFVAMWSDVGLC KKRPKP	bovine
MVKSHIGSWILVLFVAMWSDVGLC KKRPKP	sheep
MVKSHIGSWILVLFVAMWSDVGLC KKRPKP	goat
--MAHLGYWMLLLFVATWSDVGLC KKRPKP	rabbit
--MANLGYWLLALFVTTCTDVGLC KKRPKP	rat
--MANLSYWLLALFVATWTDVGLC KKRPKP	Chinese hamster
MVKSHIGSWILVLFVAMWSDVGLC KKRPKP	cat
MVKSHIGSWILVLFVAMWSDVGLC KKRPKP	dog
MVKSHIGGWILVLFVAAWSDIGLC KKRPKP	pig
Signal sequence	NLS-like sequence

Fig. 1

(57) **Abstract:** -1- ABSTRACT The present invention concerns a prion protein derived cell penetrating peptide (PrP-CPP) with binding affinity for PrP^{Sc}, capable of reducing the PrP^{Sc} level in cells. It also concerns related uses, and detection methods outlining from these PrP-CPPs. 5

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Prion protein derived cell penetrating peptides and their uses

Field of the Invention

The present invention generally relates to a prion protein derived cell penetrating peptide (PrP-CPP) with binding affinity for PrP^{Sc}, use thereof for the manufacture of a diagnostic agent to diagnose or detect a prion disease, as well as a method to detect the PrP^{Sc} level in a cell.

Background of the Invention

Prions and Prion Diseases

Prions are infectious proteins that are associated with spongiform encephalopathies, or prion diseases, which are a group of fatal and incurable neurodegenerative conditions occurring in humans and other mammals. As a disease hallmark, the endogenous cellular prion protein (PrP^C) becomes misfolded into a pathogenic amyloidogenic protein isoform called scrapie (PrP^{Sc}). The conformation of PrP^{Sc} makes it largely resistant to cellular degradation and PrP^{Sc} accumulates in the central nervous system and is in many instances deposited as aggregated prion plaques. Neuronal apoptosis and a resulting neurodegeneration are prominent features of prion disease pathogenesis. Big vacuoles are formed in axonal neurites and whole neurons are also degenerating, leaving empty spaces as a result. The accumulation of PrP^{Sc} is accompanied by severe astrogliosis and microglial cells are also constituents of the plaques.

Spongiform encephalopathies are the only known group of diseases containing infectious as well as genetic (heritable and sporadic) cases. Sporadic cases of Creutzfeldt-Jakob disease (CJD) are the most common of all the human prion diseases. Iatrogenic cases of infectious CJD have been documented to derive from blood transfusions, corneal- or other tissue-transplantations or administration of pituitary gland hormones. The source of infectivity in all such cases has been persons suffering from a non-identified human TSE. The incidence of all different types of CJD cases combined is only

around $1.3/10^6$ per year. Gerstmann-Sträussler-Scheinker (GSS) disease occurs in persons with an apparent hereditary predisposition, and Familial Insomnia (FI) are other human spongiform encephalopathies. Prion diseases occurring amongst other mammals include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Chronic wasting disease (CWD) in deer and elk populations.

The prion diseases captured the public attention with the emergence of the BSE-, or Mad-cow disease", epidemic in the 90's, and later with the appearance of variant Creutzfeldt-Jakob disease (vCJD) in humans, a novel form of CJD linked to dietary exposure of BSE. In total, about 200 cases of vCJD had been reported worldwide to date and BSE should still be considered a threat to public health. To control this problem, a huge number of animals have been recruited by international surveillance and in the EU over 20 million cows were tested for BSE during 2005-2006. Further, risk assessments still have to be performed to evaluate the implications on human health of newly identified forms of animal prion disease such as atypical BSE (BASE) or atypical scrapie (Nor 98) (S.L. Benestad, et al. (2003) Vet Rec, 153:202-8; C. Casalone, et al. (2004) Proc. Natl. Acad. Sci. USA, 101:3065-70).

Prions, the agents that transmit infectious cases of prion diseases, are composed mainly or solely of PrP^{Sc} according to the protein only hypothesis (S.B. Prusiner (1998) Proc. Natl. Acad. Sci. USA, 95:13363-83). In sporadic or hereditary cases of prion disease, PrP^{Sc} is formed by mis-folding of PrP^{C} due to mutations in the *PRNP* gene. Infection and amplification of prions is achieved by a yet poorly understood and very much debated auto-catalytic mechanism called conversion, a process for which several models have been proposed. PrP^{Sc} interacts with PrP^{C} in a manner requiring a number of circumstances under which PrP^{Sc} transmits its pathogenic conformation onto the interacting PrP^{C} protein. It has been shown that protease resistant PrP^{Sc} can be amplified using PrP^{C} as a substrate *in vitro* confirming the pro-

tein only hypothesis (J. Castilla, et al. (2005) Cell, 121:195-206; S. Supattapone (2004) J. Mol. Med, 82:348-56).

PrP^C is evolutionally conserved and is expressed ubiquitously
5 but most prominently amongst cells of the CSN, the lymphoid
organs and immune system. The physiological role of PrP^C re-
mains elusive, especially since PrP^C knock-out mice display
only minor phenotype changes. Such mice devoid of PrP^C show
10 no major neurological and physical impairment except altera-
tions in synaptic function altered sleep regulation indica-
tiong a synaptic function for this protein (H. Bueler, et
al.(1992) Nature, 356:577-82). Also, these mice are not sus-
ceptible to prion infection again confirming the protein only
hypothesis. Identified ligands to PrP^C mainly belong to heat-
15 shock proteins, membrane-bound receptors and heparan sulfates
(HS) (C. I. Lasmezas (2003) Br. Med. Bull, 66:61-70). PrP^C
binds to the extracellular matrix-protein laminin and also
with the laminin receptor precursor (LRP). Binding of PrP^C to
laminin promotes neurite outgrowth in a rat hypothalamic cell
20 line and in primary neurons of rodents (E. Graner, et al.
(2000) Brain Res. Mol. Brain Res, 76:86-92). Both neuropro-
tective and neurotoxic functional properties have been as-
signed to PrP^C, suggestively by involvement of PrP^C in signal
transduction pathways (C. I. Lasmezas (2003) Br. Med. Bull,
25 66: 61-80; L. B. Chiarini, et al. (2002) EMBO J, 21:3317-26;
E. Paitel, et al. (2003) J. Biol. Chem, 278:10061-6; E.
Paitel, et al. (2004) J. Biol. Chem, 279:612-8).

As a pathological significance of prion disease, proposed
30 PrP^C functions and interactions are often disturbed. Aberrant
regulation of neuroprotective signal transduction pathways
may contribute to the disease neurodegeneration. The multi-
tude of cellular changes occurring during the prion disease
propagation might reflect loss of PrP^C function or gain of
35 PrP^{Sc} function, or a combination of both.

The human mature PrP^C consists of amino acids 23-230. Overall,
the PrP^C secondary structure contains 42% α -helix and 3% β -sheet

(see Figure 1). Residues 126-230 form a structurally stable C-terminal region whereas the N-terminal region (residues 23-125) is essentially flexible. Many functional aspects of PrP^C are associated with the N-terminal part (C. I. Lasmezas (2003) Br. Med. Bull, 66: 61-70). Residues 1-22 constitute a signal sequence promoting entry into the endoplasmic reticulum (ER), and is cleaved off before PrP^C reaches the cell surface. Residues 23-28 or 25-30, depending on species, form a basic segment known as the preoctarepeat region, since it is preceding five eight-amino acid sequence repeats. These so called octarepeats function as Cu²⁺ ion-binding sites. A C-terminal glycosylphosphatidyl-inositol- (GPI-) anchor localizes PrP^C to functionally specialized membrane domains known as lipid rafts. Interestingly, a PrP^C N-terminal region may also localize PrP^C to lipid rafts (D. R. Taylor, et al. (2005) J. Cell. Sci, 118:5141-53). The pre- and octarepeat region (amino acids 23-100) is responsible for endocytotic internalization of PrP^C. Further, two independent nuclear localization signal-like (NLS-like) domains also reside in the N-terminal region of PrP^C, although their functions remain largely unclear (Y. Gu, et al. (2003) Neurobiol. Dis, 12: 133-49).

In sporadic and familial forms of prion disorders, mutated PrP^C changes conformation into PrP^{Sc} during folding in the ER. Mutations in the *PRNP* gene cause instability in the protein structure of PrP^C. Probably an intermediary form, PrP^{*}, is reached before the conformation tips over into the PrP^{Sc} isoform. The formation of PrP^{Sc} from PrP^C is considered a structural change. Compared to PrP^C, PrP^{Sc} has a lower α -helix content (30%) but is instead composed mainly of β -sheets (43%) (Figure 1). While PrP^C is easily digested, PrP^{Sc} becomes largely insensitive to proteases, including proteinase K (PK). Only an N-terminal fraction of PrP^{Sc} is removed by proteolytic cleavage, leaving a 27-30 kDa infectious core protein defined as PrP²⁷⁻³⁰, or simply PrP^{res}. The β -sheet content of PrP²⁷⁻³⁰ is about 50% and due to the hydrophobic properties of β -sheets, PrP²⁷⁻³⁰ has the tendency to polymerize into amyloid rods (S. B. Prusiner (1998) Proc. Natl. Acad. Sci. USA, 95: 13363-83). The autocatalytic PrP^{Sc} replica-

tion together with the slow degradation of PrP^{res} results in an exponential increase of PrP^{Sc} in neuronal tissue followed by aggregation.

5 Two different mechanisms for conversion have been proposed. The "seeding/ nucleation" model envisions a stochastic or sporadic, transient formation of PrP^{Sc} in an equilibrium almost entirely shifted towards the PrP^C conformation (K. Abid and C. Soto (2006) Cell Mol. Life Sci, 63: 2342-51; S. B. Prusiner (1991) Harvey Lect, 87:85-114; S. B. Prusiner (1998) Proc. Natl. Acad. Sci. USA, 95: 13363-83). The PrP^{Sc} conformation would only be stabilized by formation of ordered aggregates and PrP^{Sc} oligomers would recruit monomeric, transiently formed, unstable PrP^{*} shifting the equilibrium towards the PrP^{Sc} isoform. The "template assisted/refolding" model proposes that PrP^{Sc} contains a blueprint instruction applied onto PrP^C via a reaction catalyzed by a yet unidentified cofactor-X interacting with both isoforms (K. Abid and C. Soto (2006) Cell Mol. Life Sci, 63:2342-51). PrP^C transformation would involve a conformational intermediate, followed by the formation of a PrP^{Sc} dimer.

Intracellular accumulation of PrP^{Sc} is found partly in late endosomes and lysosomes of infected cells. Presumably, the endocytic pathway and/or recycling of PrP^C are involved in the synthesis of PrP^{Sc}. Localization of PrP^C to rafts is critical for its conversion into PrP^{Sc} and for prion infection. Presumably, the gradually lowered pH in the endocytic pathway of the recycling PrP^C is important for the prion conversion event (D. R. Borchelt, et al. (1992) J. Biol. Chem, 267: 16188-99; D. R. Taylor et al. (2005) J. Cell Sci, 118: 5141-53; M. Vey, et al. (1996) Proc. Natl. Acad. Sci. USA, 93: 14945-9). Several cellular components have been proposed to be necessary for conversion to occur. As such, cell surface heparan sulfate (HS) carbohydrate chains seem to function as important conversion mediators. HS is found attached to proteins named heparan sulfate proteoglycans (HSPGs) and functionally. The cell surface HSPGs seem to play a role in

PrP^C internalization (A. Taraboulos, et al. (1992) Mol. Biol. Cell, 3: 851-63). *In vitro* conversion assays has shown that presence of RNA molecules as well as heparan sulfate promotes formation of PrP^{Sc} from PrP^C (S. Supattapone (2004) J. Mol. Med., 82: 348-56). Interestingly, divergent results have been shown regarding the role of divalent metal ions for conversion *in vitro* (N. H. Kim, et al. (2005) Faseb J, 19: 783-5; N. R. Orem, et al. (2006) J. Neurochem, 96: 1409-15).

10 Cell Penetrating Peptides

Several peptides have been demonstrated to translocate across the plasma membrane of eukaryotic cells by a seemingly energy-independent pathway. These peptides are defined as cell-penetrating peptides (CPPs) and have been used successfully for intracellular delivery of macromolecules with molecular weights several times greater than their own. (M. Lindgren et al, 2000, Cell-penetrating peptides; TIPS, Vol.21, pg. 99-103). Cellular delivery using these cell-penetrating peptides offers several advantages over conventional techniques. It is non-invasive, energy-independent, is efficient for a broad range of cell types and can be applied to cells en masse. Furthermore, it has been found that for certain types of CPPs, cellular internalisation occurs at 37°C, as well as at 4°C, and that it cannot be saturated.

Many different CPP-internalization mechanisms have been proposed and also, a CPP may exhibit different entry mechanisms with or without attached cargo or in different model systems. Association with membrane phospholipids and/or HSPGs presumably initiates the CPP translocation. Also, cell penetration seems to involve lipid rafts, since cholesterol depletion reduces CPP internalization (M. Magzoub and A. Gräslund (2004) Q Rev. Biophys., 37:147-95). CPPs include helical peptides of amphipathic nature like transportan, as well as cationic arginine-rich peptides such as penetratin.

Interestingly, peptides derived from the prion protein N-terminus are CPPs (see Figure 1 for sequences). The prion protein derived CPPs (PrP-CPPs) consist of the N-terminal signal peptide comprising residues 1-22 in mouse (m) PrP and 1-24 in bovine (b) PrP, coupled to sequences 23-28 and 25-30 respectively, which in each case constitutes one of the two NLS-like sequences found in PrP^C (P. Lundberg, et al. (2002) Biochem. Biophys. Res. Commun, 299:85-90; M. Magzoub, et al. (2006) Biochem. Biophys. Res. Commun, 348:379-85).

The PrP-CPPs have been found to transport hydrophilic cargoes across cell membranes. Membrane translocation of PrP-CPPs is described as a lipid raft dependent uptake by macropinocytosis. When fluorescein-coupled mPrP₁₋₂₈ was added to mouse neuroblastoma N2a cells, the peptide was found in endosomes or in a perinuclear pattern coinciding with the Golgi and as a diffuse cytoplasmic localization. Likewise, fluoresceinyl-labelled bPrP₁₋₃₀ translocated in a similar pattern into CHO cells (P. Lundberg, et al. (2002) (P. Lundberg, et al. (2002) Biochem. Biophys. Res. Commun, 299:85-90; M. Magzoub, et al. (2006) Biochem. Biophys. Res. Commun, 348:379-85). Membrane perturbation studies in a lipid vesicle model system suggest that the PrP-CPPs cause pore formation. Macropinocytosed CPPs may escape endosomes and avoid lysosomal degradation since a transmembrane pH gradient imitating the one arising in endosomes *in vivo*, showed to elevate escape of bPrP₁₋₃₀ from lipid vesicles (M. Magzoub, et al. (2005) Biochemistry, 44: 14890-7).

Prior Art

WO 03/106491 A2 discloses methods for predicting or designing, detecting, and/or verifying a novel CPP. P. Lundberg, et al. (2002) Biochem. Biophys. Res. Commun, 299:85-90 Cell membrane translocation of the N-terminal (1-28) part of the prion protein, discloses that the N-terminal part of the prion protein is responsible for cell membrane translocation. A. L. Lau, et al. (2007) Proc. Natl. Acad. Sci. USA, 104: 11551-6. Characterization of prion protein (PrP)-derived peptides that discriminate

full-length PrP^{Sc} from PrP^C, discloses that residues 23-30 are important for the binding of PrP₁₉₋₃₀ to PrP^{Sc}.

Summary of the Invention

5

The present inventors have surprisingly found that cell penetrating peptides derived from the prion protein can antagonize prion infection. The exact mechanism of the antagonist action is not known. However, without being bound by any particular theory, it is possible that the binding of PrP-CPP to PrP^{Sc} inhibits the PrP^{Sc} mediated conversion of PrP^C to PrP^{Sc}. It is also possible that the binding makes PrP^{Sc} more readily degradable.

15 Therefore, in a first aspect, the invention relates to a prion protein derived cell penetrating peptide (PrP-CPP) with binding affinity for PrP^{Sc}, wherein the PrP-CPP reduces the PrP^{Sc} level in cells. In an embodiment of this first aspect, the PrP-CPP is mPrP₁₋₂₈ or bPrP₁₋₃₀.

20

In another aspect, the invention relates to the use of a PrP-CPP with binding affinity for PrP^{Sc}, for reducing the PrP^{Sc} level in a mammal.

25 In yet other aspects, the invention relates to the use of a PrP-CPP with binding affinity for PrP^{Sc} for the manufacture of a diagnostic agent to diagnose or detect a prion disease, and to this diagnostic agent.

30 In yet another aspect, the invention relates to the use of a PrP-CPP with binding affinity for PrP^{Sc} as an antigen for the selection, design or production of an immune epitope molecule.

In yet another aspect, the invention relates to a method for
35 reducing the PrP^{Sc} level in a cell, comprising administering a prion protein derived cell penetrating peptide (PrP-CPP) with binding affinity for PrP^{Sc}.

Brief Description of the Drawings

Figure 1 shows the alignment of PrP amino acid sequences from human (SEQ ID NO. 1), mouse (SEQ ID NO. 2), bovine (SEQ ID NO. 3), sheep (SEQ ID NO. 4), goat (SEQ ID NO. 5), rabbit (SEQ ID NO. 6), rat (SEQ ID NO. 7), chinese hamster (SEQ ID NO. 8), cat (SEQ ID NO. 9), dog (SEQ ID NO. 10) and pig (SEQ ID NO. 11). The boxes highlighted are according to the mouse sequence, the highly conserved signal peptide sequences at amino acids 1-22 (in light grey) as well as the NLS-like sequence at amino acids 23-28 (in dark grey).

Figure 2 shows the different PrP^C and PrP^{Sc} prion protein isoform structures. The GPI-anchor is pictured in black, where the conformational change into PrP^{Sc} changes PrP to become resistant to removal of the GPI-anchor by cleavage with phosphoinositol specific phospholipase C (PIPLC).

Figure 3. shows the NMR solution structure of bPrP₁₋₃₀ in DHPC micelles as previously presented by H. Biverstahl, et al. (2004) Biochemistry, 43: 14940-7. The structure is an ensemble of 22 structures superimposed on backbone atoms in residues Ser8-Val21. The helical region, Ser8-Val21, is indicated by a light grey color, while the termini are in dark grey.

Figure 4 shows the level of PrP^{Sc} in ScGT1-1a and ScGT1-1b cells after treatments with mPrP₁₋₂₈. A) Western blot of PrP levels in whole cell lysates from ScGT1-1a cells after treatment with 2 µM mPrP₁₋₂₈ for 3, 5 or 8 days (d) and corresponding treatment with 2 µM PPS compared to untreated control (UT). The PrP^{Sc} specific bands (e.g. notably at 18 kDa) show a time-dependent reduction in intensity. B) Western blot showing a time-dependent reduction of proteinase K resistant PrP^{Sc} in ScGT1-1 cells by treatment with 2 µM mPrP₁₋₂₈. The cell lysates from PrP-CPP treated or untreated (UT) ScGT1-1a or ScGT1-1b cell cultures were treated with PK (+) prior to detection of PrP^{Sc}. C) Graph showing the relative ratios in %

of PrP^{Sc} in ScGT1-1a or ScGT1-1b cells after treatment with 2 μ M mPrP₁₋₂₈ compared to untreated control (UT). All treatments show statistically significant reduction of PrP^{Sc} levels (*P<0.05 **P<0.01).

5

Figure 5 A) shows the protein level of PrP^C in GT1-1 cells after treatments with 2 μ M mPrP₁₋₂₈ as analyzed by western blot probed with a polyclonal PrP antibody. The cell lysates from PrP-CPP treated or untreated (UT) GT1-1 cell cultures were separated on a 12% SDS-PAGE under reducing conditions. B) Graph showing the relative ratios as % of PrP^C in GT1-1 cells after treatment with 2 μ M of mPrP₁₋₂₈ compared to untreated control (UT). No statistically significant differences in PrP^C levels were found.

15

Figure 6 shows the level of PrP^{Sc} in ScGT1-1 cells after treatments with bPrP₁₋₃₀. A) Western blot of PrP levels in whole cell lysates from ScGT1-1a and ScGT1-1b cells after treatment with 2 μ M bPrP₁₋₃₀ for 3, 5 or 8 days (d) compared to untreated control (UT). The PrP^{Sc} specific bands (e.g. notably at 18 kDa) show a time-dependent reduction in intensity. B) Western blot showing a time-dependent reduction of proteinase K resistant PrP^{Sc} in ScGT1-1b cells by treatment with 5 μ M bPrP₁₋₃₀. The cell lysates from PrP-CPP treated or untreated (UT) ScGT1-1b cell cultures were treated with PK (+) prior to detection of PrP^{Sc}.

25

Figure 7 A) shows the protein level of PrP^C in GT1-1 cells after treatments with 2 μ M bPrP₁₋₃₀ as analyzed by western blot probed with the polyclonal PrP antibody. B) Graph showing the relative ratios as % of PrP^C in GT1-1 cells after treatment with 2 μ M of bPrP₁₋₃₀ compared to untreated control (UT). No statistically significant differences in PrP^C levels were found.

35

Figure 8 shows dose-response curves displaying that treatment with the PrP-CPPs mPrP₁₋₂₈ and bPrP₁₋₃₀ significantly reduced PrP^{Sc} levels in ScGT1-1b cells in a dose-dependent manner.

PrP^{Sc} levels were set in % relative to untreated controls. Concentrations in μ M of peptide were tested as presented in table 1 and is set as $\log^{10}$. IC₅₀ for the PrP^{Sc} reduction after 8 days of treatment with mPrP₁₋₂₈ was calculated to be
5 0.3 $\pm$ 0.07 μ M. IC₅₀ for the PrP^{Sc} reduction after 8 days of treatment with bPrP₁₋₃₀ was calculated to be 3.3 $\pm$ 0.68 μ M.

Figure 9 shows the level of PrP^{Sc} in ScGT1-1b cells after treatments with mPrP₂₃₋₂₈ or mPrP₂₃₋₅₀ as analyzed by western
10 blot probed with the polyclonal PrP antibody. The cell lysates from PrP-peptide treated or untreated (UT) ScGT1-1b cell cultures were treated with proteinase K (+) or not (-). The three western blots show whole cell PrP (-PK) and PrP^{Sc} (+PK) levels after 8 days of treatment with 2, 5 or 10 μ M mPrP₂₃₋₅₀
15 or mPrP₂₃₋₂₈ as indicated. No statistically significant effects on PrP^{Sc} levels were produced by any of these treatments.

Figure 10 shows graphs of the level of PK resistant PrP^{Sc} in ScGT1-1b cells or level PrP^C protein in GT1-1 cells after
20 treatment with 5 or 10 μ M PrP peptides. A) Graph displaying the relative ratios in % of PrP^{Sc} in ScGT1-1a or ScGT1-1b cells after treatment with mPrP₁₋₂₈, bPrP₁₋₃₀, mPrP₂₃₋₅₀ or mPrP₂₃₋₂₈ compared to untreated controls (UT). Only the treatments with mPrP₁₋₂₈ or bPrP₁₋₃₀ produce statistically significant
25 cant reduction of PrP^{Sc} levels (*P<0.05 **P<0.01). E) Graph showing the relative ratios as % of PrP^C in GT1-1 cells after treatment with mPrP₁₋₂₈, bPrP₁₋₃₀, mPrP₂₃₋₅₀ μ M mPrP₂₃₋₂₈ compared to untreated control (UT). No statistically significant differences in PrP^C levels were found after treatments with any
30 of the peptides.

Figure 11 shows western blots detecting the levels of PrP in whole cell lysates (-) or PrP^{Sc} in PK digested lysates (+) from GT1-1 cells incubated with 0.05% of RML brain homoge-
35 nate. Cells were extracted 20 days post infection or 35 days post infection. During the 3 days of RML infection, cells were simultaneously treated with 10 μ M of mPrP₁₋₂₈, bPrP₁₋₃₀ or mPrP₂₃₋₅₀. To RML-infected control cells (C) addition of PBS

was made. Non-infected GT1-1 cells served as infection control. Blots were probed with the polyclonal PrP antibody. A) After 20 days of cultivation post infection, levels of PrP^{Sc} were significantly lower in cultures to which mPrP₁₋₂₈ or bPrP₁₋₃₀ had been added during the RML infection compared to the control, or compared to the cultures treated with mPrP₂₃₋₅₀ during RML infection. B) When the cultures tested in A) were passaged for additional 3 passages (35 days post infection) no differences in PrP^{Sc} levels could be detected.

Figure 12 shows a graph of the level of PK resistant PrP^{Sc} in GT1 cells 20 days after that the cell cultures were incubated with 0.05% of RML brain homogenate and simultaneously treated with 10 µM of mPrP₁₋₂₈, bPrP₁₋₃₀ or mPrP₂₃₋₅₀. The levels of accumulated PrP^{Sc} were significantly lower in cultures where GT1-1 cells had been treated with mPrP₁₋₂₈ or bPrP₁₋₃₀ during RML infection (**P<0.01). Treatment with mPrP₂₃₋₅₀ during RML infection produced no significant protection against PrP^{Sc} accumulation.

Figure 13 shows that PrP^C or PrP^{Sc} binding to heparin is not affected by presence of mPrP₁₋₂₈. Western blot detecting PrP in heparin conjugated-agarose (HA) precipitates made on GT1-1 or ScGT1-1b cell lysates. Precipitation of PrP^C was detected from GT1-1 cells. Precipitates from ScGT1-1b cells were exposed to PK degradation before SDS-PAGE (+PK), showing PrP^{Sc} binding to HA (ScGT1-1b +PK lanes). Preincubation of HA with 200 µM of mPrP₁₋₂₈ showed no effect on levels of precipitated PrP^C from GT1 or PrP^{Sc} (+PK) from ScGT1-1b (lanes HA preinc. mPrP₁₋₂₈) compared to controls (Control 1). Preincubation of lysates with 100 µM of mPrP₁₋₂₈ prior to HA precipitation gave no effect on levels of precipitated PrP^C from GT1 or PrP^{Sc} (+PK) from ScGT1-1b (lanes lysate preinc. mPrP₁₋₂₈) compared to controls (Control 2). Final concentration of mPrP₁₋₂₈ during incubations, in samples where peptide was added, was 67 µM. For control 1, HA beads were preincubated with lysis buffer instead of mPrP₁₋₂₈ before addition of lysate and further incubation. For control 2, lysates were preincubated

with lysis buffer instead of mPrP₁₋₂₈ before addition of HA beads and further incubation. * The detection of PrP^{Sc} levels in control 2 ScGT1-1b HA-PrP^{Sc}-precipitates is faulty in this picture. However, the levels of PK-resistant PrP^{Sc} from HA-precipitates on ScGT1-1b lysates were repeatedly measured not to differ between control 1 and 2 in replicates of these experiments.

Figure 14 shows graphs of the level of PK resistant PrP^{Sc} in ScGT1-1 cells or level PrP^C protein in GT1-1 cells after 3, 5 or 8 days of treatment with indicated concentrations of non-PrP CPPs. No reproducible or consistent time-dependent reduction of PrP^{Sc} levels (ScGT1-1) or effect on PrP^C protein level (GT1-1) was detected by treatment with any of these peptides.

A) Graph showing the relative ratios in % of PrP in indicated cell lines after treatment with 6 µM 16-mere arginine (R₁₆) compared to untreated control (UT). No statistically significant differences in PrP levels were found. B) Graph showing the relative ratios in % of PrP in indicated cell lines after treatment with 1 µM of penetratin compared to untreated control (UT). No statistically significant differences in PrP levels were found. C) Graph showing the relative ratios in % of PrP in indicated cell lines after treatment with 2 µM of transportan-10 (TP-10) compared to untreated control (UT).

Levels of PrP^{Sc} were significantly lowered by 8 days of treatment with TP-10 of ScGT1-1a cells, however the opposite effect was found after 5 or 8 days of treatment with TP-10 of ScGT1-1b cells, in which the levels of PrP^{Sc} were significantly elevated (*P<0.05). No statistically significant differences in PrP^C levels were found.

Figure 15 shows dose-response curves displaying that treatment with non-PrP derived CPPs or prion protein derived peptides containing the NLS-like sequence does not significantly affect the PrP^{Sc} levels in ScGT1-1b cells. PrP^{Sc} levels were set in % relative to untreated controls. A) Dose-response curve displaying the effect of mPrP₂₃₋₂₈ on the PrP^{Sc} level in ScGT1-1b cells. Concentrations in µM of peptide were tested

as presented in table 1 and is set as $\log^{10}$ B) Dose-response curve displaying the effect of mPrP₂₃₋₅₀ on the PrP^{Sc} level in ScGT1-1b cells. Concentrations in μM of peptide were tested as presented in table 1 and is set as $\log^{10}$ C) Dose-response curves displaying the effects of penetratin and transportan-10 (TP-10) on the PrP^{Sc} level in ScGT1-1b cells. Concentrations in μM of peptide were tested as presented in table 1 and is set as $\log^{10}$ D) Dose-response curve displaying the effect of 16-mer arginine (R₁₆) on the PrP^{Sc} level in ScGT1-1b cells. Concentrations in μM of peptide were tested as presented in table 1 and is set as linear. Concentrations above 6 μM had strong toxic effects on the cell cultures.

Figure 16 shows a western blot detecting the levels of PrP in whole cell lysates (-) or PrP^{Sc} in PK digested lysates (+) from GT1-1 cells incubated with 0.05% of RML brain homogenate. Cells were extracted 20 days post infection or 35 days post infection. During the 3 days of RML infection, cells were simultaneously treated with 10 μM of mPrP₁₋₂₈, bPrP₁₋₃₀, penetratin or transportan-10 (TP-10). After 20 days of cultivation post infection, levels of PrP^{Sc} were not changed in cultures to which penetratin or TP-10 had been added during the RML infection compared to the control, or compared to the cultures treated with PrP-CPPs during RML infection in which the levels of PrP^{Sc} were significantly reduced.

Figure 17 A) Western blot of PrP from peptide-treated ScGT1-1-RML and ScGT1-1-22L cell lines. Cells were treated for 5 days with 5 μM of indicated peptide. Fresh peptide additions were made every 24th hour. B-D) Graphs showing relative levels of PrP in cells after 5 days of treatment with 5 μM of indicated peptides. B) PrP^C in GT1-1 cells, C) PrP^{Sc} levels in ScGT1-1-RML or D) ScGT1-1-22L cells. The statistical analysis has been performed by using the amount of PrP^{Sc} specific PrP divided by the total amount of PrP. $n = 3$, $**P < 0.01$ $*P < 0.05$. UT.C, untreated control; mL, mPrP_{1-28L}; mD, mPrP_{1-28D}, mDL, mPrP_{1-22D23-28L}, pen-PrP, penetratin-PrP₂₃₋₂₈.

Figure 18 Western blot of PrP from peptide-treated ScGT1-1-RML and ScGT1-1-22L cell lines. Cells were treated for 5 days with 10 μ M of indicated peptide. Fresh peptide additions was added on one occasion at day 1 (one day after seeding out of cells), medium was changed once at 72 h post peptide addition. UT.C, untreated control; mL, mPrP_{1-28L}; mD, mPrP_{1-28D}, mDL, mPrP_{1-22D23-28L}, pen-PrP, penetratin-PrP₂₃₋₂₈.

Figure 19 Western blot of PrP from peptide-treated GT1-1, ScGT1-1-RML and ScGT1-1-22L cell lines. Cells were treated for 5 days with 5 μ M of indicated peptide. Fresh peptide additions were made every 24th hour. UT, TP-10-mPrP₂₃₋₂₈; L, transportan-10-mPrP₂₃₋₂₈.

Detailed Description

This invention includes the finding that peptides derived from the prion protein can antagonize prion infection. The prion protein-derived peptides reducing the PrP^{Sc} level in prion infected GT1-1 cells include the N-terminal signal sequence and function as CPPs. mPrP₁₋₂₈ strongly reduces the PrP^{Sc} protein levels in ScGT1-1a/b cells without affecting the PrP^C protein levels in GT1-1 cells. The bovine counterpart bPrP₁₋₃₀ has the same anti-PrP^{Sc} effect as mPrP₁₋₂₈ although with a higher IC₅₀ value. Also, treatment with either mPrP₁₋₂₈ or bPrP₁₋₃₀ during RML scrapie-infection dramatically prolong the prion course of infection.

Definitions

As used herein, the term "prion protein derived" signifies a protein fragment or peptide that has an amino acid sequence that is substantially identical to at least a part of the amino acid sequence of a prion protein. Such a part typically comprises ten amino acids or more. A prion protein derived molecule can be manufactured and/or isolated using any known means in the art.

As used herein, the term "prion protein derived cell penetrating peptide", or "PrP-CPP", refers to a prion protein derived peptide with the ability to translocate across cellular membranes. It comprises the N-terminal part of the prion protein, as detailed in the description.

As used herein, the term "diagnostic agent" refers without limitations to a compound that is appropriate for application in a diagnostic purpose to diagnose or detect prion disease in a human or any other animal such as a livestock species (for example, a bovine, goat, pig, or sheep), or a species used for experimental procedures (for example mouse, hamster or rat or fruitfly) or a species used as pet (for example cat or dog).

As used herein, the term "prion disease" refers to the group of prion-mediated degenerative disorders known as spongiform encephalopathies. In humans these diseases comprise different types of Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler syndrome (GSS), fatal familial insomnia (FFI) and Kuru. Prion diseases occurring in other mammals comprise scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle, as well as prion diseases in other ruminants and cats.

As used herein, the term "treatment of prion diseases" refers without limitations to the ability a compound to prevent, diminish, or slow down the onset of, any symptom associated with prion diseases, particularly the accumulation of PrP^{Sc}.

Reduction of the PrP^{Sc} level by treatment with mPrP₁₋₂₈

Treatments with mPrP₁₋₂₈ produces a time- and dose-dependent reduction of PrP^{Sc} in both ScGT1-1a and ScGT1-1b. A statistically significant reduction of PrP^{Sc} levels in ScGT1-1b cells can be detected already after 3 days with 1 μ M of mPrP₁₋₂₈ (data not shown), whereas 0.1 μ M of mPrP₁₋₂₈ produces a significant reduction of PrP^{Sc} protein levels in ScGT1-1a/b

cells after 8 days of treatment (Fig. 8). The PrP^{Sc} reducing effect caused by mPrP₁₋₂₈ was quantitatively as efficient as the effect by PPS, which is a well established anti-prion agent (Figure 4 A). Corresponding treatments with mPrP₁₋₂₈ in GT1-1 cells resulted in no significant changes in PrP^C protein levels (Figure 5 B and 10 B). Cultivation of ScGT1-1b cells in the presence of 0.5 μ M mPrP₁₋₂₈ showed to retain the PrP^{Sc} level at an approximatively 60 % reduced level for up to 30 days. If mPrP₁₋₂₈ treatment was aborted, levels of PrP^{Sc} returned to the ones found in untreated ScGT1-1b cells (data not shown).

Likewise, treatments with mPrP₁₋₂₈ in the D-configuration produces a reduction of PrP^{Sc} in ScGT1-1 cells infected with either the RML or 22L strain of prions (ScGT1-1-RML and ScGT1-122L) (Figure 17 A, C and D). The reduction of PrP^{Sc} levels in RML or 22L infected ScGT1-1 cells is as efficiently achieved by using the D-form of mPrP₁₋₂₈ as the regular L-form. The PrP^{Sc} reducing effect caused by mPrP₁₋₂₈ all-D was quantitatively as efficient as the effect by the peptide in L-configuration. Further, the same PrP^{Sc}-reducing effect can be obtained by administration of a mPrP₁₋₂₈ peptide where residues 1-22 is in the D-configuration and residues 23-28 are in the L-configuration (mPrP_{1-22 (D) 23-28 (L)}). Corresponding treatments with mPrP₁₋₂₈ all-D or mPrP_{1-22 (D) 23-28 (L)} in GT1-1 cells resulted in no significant changes in PrP^C protein levels (Figure 17 B).

Reduction of the PrP^{Sc} level by treatment with bPrP₁₋₃₀

2 μ M treatments with mPrP₁₋₂₈ result in significantly reduced the level of PrP^{Sc} in ScGT1-1a/b cells already after 3 days of treatment (Figure 6 A). 8 day treatments of ScGT1-1b cells with the peptides mPrP₁₋₂₈, mPrP₂₃₋₅₀, bPrP₁₋₃₀ at the final concentrations 5 or 10 μ M, or with mPrP₂₃₋₂₈ at 25 or 50 μ M show that these concentrations of bPrP₁₋₃₀ significantly reduced the levels of PrP^{Sc} similar to the effects by treatment with mPrP₁₋₂₈ as mentioned above (Figure 6 B and 10 A). Treatment of GT1-1

cells with peptides in these concentrations show no significant cellular loss nor any effect on PrP^C protein (Figure 10 B). None of the other tested peptides produce any effect on PrP^{Sc} levels at these concentrations (Figure 9 and 10 A).

5

Prolongation of the RML course of infection by treatment with PrP-CPPs

GT1-1 cells can be infected with scrapie by incubation with
10 RML mouse brain homogenate. In cells as well treated with 10
μM of either mPrP₁₋₂₈, bPrP₁₋₃₀, mPrP₂₃₋₅₀, or with 50 μM of
mPrP₂₃₋₂₈ during the 3 days of RML treatment, cultures which
had been simultaneously incubated with either mPrP₁₋₂₈ or
bPrP₁₋₃₀ showed significantly lower levels of PrP^{Sc} after 20
15 days of cultivation post the 3 days of RML treatment (Figure
11 A and 12). The RML course of infection is not affected by
any of the other peptides mentioned (Figure 11A, 12 and 16).

Recently, two prion protein-derived peptides, PrP₁₉₋₃₀ and
20 PrP₁₀₀₋₁₁₁, were found to interact more specifically with PrP^{Sc}
than with PrP^C (A. L. Lau, et al. (2007) Proc. Natl. Acad.
Sci. USA, 104: 11551-6). The binding activity of these pep-
tides was found not to correlate with hydrophobic nor polar
interactions. However, the interaction was found to be af-
25 fected by amino acid composition and foremost by the positive
charge of the two peptides. The finding that PrP₁₉₋₃₀ bind
specifically to PrP^{Sc} suggests a direct interaction between
mPrP₁₋₂₈ or bPrP₁₋₃₀ and PrP^{Sc}. This specific interaction poten-
tially is responsible for the reducing effect these CPPs have
30 on the PrP^{Sc} levels in ScGT1-1 cells. The peptide PrP₁₉₋₃₀ was
not found to interact with PrP^C. This may correspond with our
present data that the PrP-CPPs do not affect the PrP^C protein
level in GT1-1 cells.

35 Specificity of PrP-CPPs for reducing the PrP^{Sc} level

The reducing effect on PrP^{Sc} levels is specific for the CPPs
derived from the N-terminus of the prion protein. Different

treatments with a broad range of other CPPs like TP-10, penetratin or R₁₆ had no effect on the PrP^{Sc} level (Figure 14, 15 B and C and 16). Further, treatment with a peptide corresponding to one of the NLS-like sequences in the protein protein,

5 mPrP₂₃₋₂₈, had no effect on the PrP^{Sc} level (Figure 10 A and 15 A). Likewise, treatment with the peptide mPrP₂₃₋₅₀ i.e. the pre-octarepeat sequence, showed no effects on the amount of PrP^{Sc} (Figure 10 A and 14 A). In the report A. L. Lau, et al. (2007) Proc. Natl. Acad. Sci. USA, 104: 11551-6 successive shortening

10 of the peptide PrP₁₉₋₃₀ identified residues PrP₂₃₋₃₀ as the peptide core important for binding to PrP^{Sc}. Nevertheless, we here show that even if a peptide contains this sequence, it is still not enough for reducing the PrP^{Sc} level in prion-infected cells. Shortening of the mPrP-CPP into mPrP₁₂₋₂₈ rendered no effect on the PrP^{Sc} levels in ScGT1-1-RML or ScGT1-122L cell cultures after 5 day treatments (Figure 19). Thus, the β -

15 structure formation and membrane associative elements included in mPrP₁₋₂₈ by the sequence 1-22 is necessary for the anti-prion action, with residues 1-11 being critical for this function, whereas residues 23-28 potentially is promoting the actual interaction with PrP^{Sc}. The signal sequence works as the label for cellular localization, whereas the NLS-like sequence promotes interaction with PrP^{Sc}.

25 In both RML and 22L-infected GT1-1 cell cultures, both the D- and L-configuration of mPrP₁₋₂₈, as well as a combination of amino acid configurations, peptide mPrP_{1-22 (D) 23-28 (L)}, produced an efficient reduction of PrP^{Sc} levels (Figure 17). This effect is very potent, since a single addition of peptide in

30 either conformation produces strong reduction of PrP^{Sc} already after 5 days (Figure 18). That there is no conformational requirement for the PrP-CPP anti-prion action strongly suggests that the mechanism for this effect is not due to a chiral /configuration-specific interaction with PrP^{Sc} or any

35 other component of the conversion-aiding platform.

Interestingly, in another recent study recombinant peptide aptameres with affinity for PrP were shown to abolish PrP^{Sc}

conversion when added to prion infected neuroblastoma cells (ScN2a) (S. Gilch, et al. (2007) J. Mol. Biol., 371: 362-73). Further, two of these peptide aptameres were also found to interfere with PrP^{Sc} formation when expressed in the secretory pathway of ScN2a cells. Potentially, these peptide aptameres inhibit PrP conversion in different manners when exogenously added to the cell culture, or when endogenously expressed. As CPPs, mPrP₁₋₂₈ and bPrP₁₋₃₀ may reach multiple destinations where interference with PrP^{Sc} could occur.

10

Strong evidence suggests that the endocytic pathway has an important role in the formation of PrP^{Sc}. We hypothesize that the PrP-CPPs inhibit conversion along this pathway. It has been suggested that cationic domains of PrP^C may be important in the interaction between PrP^C and PrP^{Sc} since cationic compounds are promoting a reduction of PrP^{Sc} levels in prion infected cell cultures (S. A. Priola, et al. (2000) Science, 287: 1503-6; K. F. Winklhofer and J. Tatzelt (2000) Biol. Chem., 381: 463-9). Binding of the positively charged PrP-CPPs to PrP^{Sc} could block the interaction between PrP^{Sc} and PrP^C, inhibiting conversion. Nevertheless, the anti-prion properties of mPrP₁₋₂₈ and bPrP₁₋₃₀ are not due strictly to their cationic properties. Other positively charged CPPs tested, such as R16, showed no effects on the PrP^{Sc} levels, neither did PrP-derived peptides mPrP₂₃₋₂₈ and mPrP₂₃₋₅₀, which include the same basic residues as the PrP-CPPs.

Treatments of ScGT1-1-RML or ScGT1-122L cell cultures with conventional cell penetrating peptides constructed to contain the NLS-like mPrP₂₃₋₂₈ sequence showed no effect on the PrP^{Sc} levels. Penetratin-mPrP₂₃₋₂₈ is suspected to penetrate into cells via a nonspecific internalization mechanism. Thus, when the mPrP₂₃₋₂₈ sequence, presumably being the PrP^{Sc}-interacting part of mPrP₁₋₂₈, is taken up into the cytosol, there is no potent anti-prion effect (Figure 17). Similarly, the CPP transportan-10 has been suggested to translocate in a nonspecific manner as well as through induction of endocytosis. When mPrP₂₃₋₂₈ was coupled to this construct (transportan-10-

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mPrP₂₃₋₂₈) no/a faint reducing effect on the PrP^{Sc}-levels could be detected after treatment of ScGT1-1-RML and ScGT1-1-22L cells (Figure 19). This further strengthens the suggestion that the PrP-CPPs assert their anti-prion effects in a specific subcellular compartment which they reach through an internalization mechanism specific for the PrP-CPPs.

However, additional and/or alternative mechanisms are possible for explaining the anti-prion effect of mPrP₁₋₂₈ and bPrP₁₋₃₀. The PrP-CPPs are believed to enter the cell through initiated macropinocytosis. Inside the endosome, the PrP-CPPs may become shuttled to the golgi and through vesicle transport circle in the secretory pathway. In these compartments, the PrP-CPPs may associate to PrP^C and by this promote similar effects as described for the peptide aptameres endogenously expressed in the secretory pathway, which suggestively inhibit PrP^{Sc} formation through overstabilization of PrP^C. However, our present data does not indicate any interactions between the PrP-CPPs and PrP^C.

It is possible that introduction of the PrP-CPPs could alter the endocytotic fate of rafts and the resident PrP by the initiation of lipid-raft mediated macropinocytosis. This could change the propensity of PrP^C to become available for conversion or it may elevate the degradation of PrP^{Sc}. Another possible mechanism underlying their anti-prion properties is that the binding of the PrP-CPPs to PrP^{Sc} by itself may render PrP^{Sc} more susceptible to lysozomal degradation in a yet unknown manner.

Further, there are several conceivable ways in which HS can be involved in the anti-PrP^{Sc} function of mPrP₁₋₂₈ and bPrP₁₋₃₀. Since HS chains are shown to facilitate PrP^{Sc} conversion *in vivo* and *in vitro*, the PrP-CPPs also interacting with HS might interfere with PrP^{Sc} formation by competitive binding to cell surface HSPGs. However, our results shows that the direct PrP^C or PrP^{Sc} binding to heparin-conjugated agarose is not affected by presence of mPrP₁₋₂₈ (Figure 13). Hence, the

PrP-CPPs are not likely to affect the PrP^{Sc} levels in ScGT1-1c by competitive inhibition of the PrP^{Sc} association with cell surface HS. Suggestively, if an interaction between the PrP-CPPs and HS indeed is responsible for counteracting PrP^{Sc} conversion, mPrP₁₋₂₈ and bPrP₁₋₃₀ may introduce steric hindrance into the PrP^C-HS-PrP^{Sc} complex disabling the putative function of this as a conversion platform.

Prion disease therapeutics

The methods and compositions of the invention provide means for evaluation of whether an individual suffers from prion disease. As noted above, the PrP-CPP might be binding to a part of PrP^{Sc} in a specific manner. In this case, the PrP-CPPS may be used as diagnostic tools in order to establish prion infection in a individual or sample.

The methods and compositions of the invention also provide means for treating or preventing prion diseases in mammals including, without limitation, humans, cattle and sheep. As noted above, the PrP-CPP might be binding in a competitive manner to a part of PrP^{Sc} or to any of the cellular PrP^{Sc} interaction molecules critical in the PrP^C to PrP^{Sc} conversion reaction. It is also possible that the PrP-CPPs via a direct interaction with PrP^{Sc} contribute to a change in physico-chemical properties of PrP^{Sc} such as the hydrophobicity, tendency to aggregate or susceptibility to cellular degradation. In any case, the PrP-CPPs may potentially be used in treatments for prion diseases as antagonists disrupting, suppressing, debilitating or inhibiting PrP^C to PrP^{Sc} conversion.

Evaluation of whether a PrP-CPP or any compound designed thereof would support protection against development of a prion disease *in vivo* involves using an animal known to develop such a disease (e. g., prion infected mouse or hamster). A prion-infected animal should be treated with the test compound according to standard methods and the onset or progression of prion related illness compared to untreated

control animals should be detected for indication of protection by the compound. The PrP-CPP or test compound designed thereof may be administered to a previously prion infected animal or, alternatively, the test compound may be tested for neutralization of prion infection by pre-incubating the infectious material with the compound prior to administration to a healthy test animal.

An anti-prion therapeutic often needs to be administered with a pharmaceutically-acceptable diluent, carrier, or excipient. As administration routes the following examples can be employed; intravenous, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. However, in most of these administration manners the therapeutic compound is susceptible to inactivation due to degradation. The PrP-CPPs readily crosses cellular membranes and reports suggest that CPPs might also be able translocate across the blood-brain barrier (M. Adenot, et al. (2007) *Chemotherapy*, 53: 73-6; M. Adenot, et al. (2007) *Chemotherapy*, 53: 70-2). Hence, the intravenous route of administration may be preferable for the PrP-CPPs in therapeutical trials.

The methods of the present invention may be used to diagnose prion infection or to reduce or prevent the disorders described herein in any animal, for example, humans, domestic pets, or livestock. Our results show that the reduction of the PrP^{Sc} level in scrapie infected mouse neuronal cells has a lower IC value for mouse PrP-CPP than for a bovine PrP-CPP (Figure 8). Thus, when a animal is diagnosed or treated using a PrP-CPP, the employed peptide may preferably be specific for that species.

The PrP-CPPs strongly reduce the level of PrP^{Sc} in prion infected cells, and also interferes with the prion course of infection. That the PrP-CPPs may enter and assert their anti-prion function through pathways not targeted by other sub-

stances known to affect PrP^{Sc} levels opens new possibilities for treatment. Suggestively, a combinatory treatment with mPrP₁₋₂₈ and a substance such as PPS may yield a synergistic effect, lowering the PrP^{Sc} levels below the threshold for PrP^{Sc} clearance *in vivo*.

Immunization using PrP-CPPs

Monoclonal antibodies may be generated according to standard methods. Monoclonal antibodies are generally prepared using the method of G. Kohler and C. Milstein (1975) *Nature*, 256: 495-7, or a modification thereof.

Typically, immune response should be enhanced with repeated booster injections, at intervals of 3 to 8 weeks. Mice are immunized with mouse PrP-CPP in complete Freund's adjuvant, each injection consisting of approximately 200 µg of the PrP-CPP. Splenocytes from these mice may be fused to other cell lines to generate specific hybridoma clones. Hybridoma supernatants may then be screened by ELISA to confirm supernatants reactive to PrP-CPP or to the entire prion protein sequence to confirm the success of the immunization. Polyclonal antibodies may be prepared according to standard methods by immunization of rabbits with PrP-CPP. More specifically, to generate polyclonal antibodies to PrP-CPPs could be an alternative interesting for the use of antibodies as diagnostic tools in prion detection, diagnostics and decontamination.

Purification of antibody and the production of anti-antibodies specific for PrP^{Sc}

Total mouse IgG from PrP-CPP immunized mice could be purified from serum using affinity chromatography. Fractions could then be analyzed on SDS-PAGE. Potentially, vaccines may be generated outlining from these antibodies according to standard methods. Humanized forms of e.g. murine antibodies may be constructed and characterized according to standard methods for this purpose since humanized antibodies are less likely to be

immunogenic and are useful in passive immunotherapies (N. R. Gonzales, et al. (2005) Tumour Biol, 26: 31-43).

5 Techniques for raising anti-idiotypic antibodies are known in the art. See, e. g. J. M. Grzych, et al. (1985) Nature, 316: 74-6; F. G. Uytdehaag and A. D. Osterhaus (1985) J. Immunol., 134: 1225-9.

10 Typically, the antigen-binding (Fab) fragment from a monoclonal antibody specifically targeting the PrP-CPP could in turn be used to immunize mice subsequently used to generate hybridomas potentially producing antibodies with specific binding to PrP^{Sc}. This second set of antibodies are labelled anti-idiotypic. Once screened for and produced, the PrP-CPP
15 anti-idiotypic antibodies should be tested for specific PrP recognition by immunoprecipitation and western blot analysis. These PrP-CPP anti-idiotypic antibodies should be investigated as reagents suitable for use in PrP^{Sc} analyses and for diagnostic use.

20

Prion decontamination

The PrP-CPPs and compositions described herein are useful for the detection and/or decontamination of material that are known
25 or suspected of being contaminated by prions. In particular, PrP-CPPs may be incubated with biological samples to complex with, and thereby inhibit the infectious potential of PrP^{Sc}.

Material and methods

30 Materials

Table 1 shows amino acid sequences for the peptides screened for effects on PrP^C (GT1-1) and PrP^{Sc} (ScGT1-1a/b) protein levels. Treatments with all indicated concentrations were
35 carried out during 3, 5 or 8 days before cell harvest and analysis. The peptide sequences presented include acetylation (Ac) and/or amidation (NH₂). * Mouse peptide PrP₁₋₂₈ was tested in the L configuration for all stated concentrations, and in

the D configuration of amino acids in concentrations indicated *. Also tested in concentrations indicated * was a mPrP₁₋₂₈ peptide with amino acids 1-22 in the D configuration followed by amino acids 23-28 in the L-configuration (mPrP_{1-22(D)23-28(L)}). ^a treatments with *all*-D mPrP₁₋₂₈, mPrP_{1-22(D)23-28(L)}, and other indicated peptides were carried out for 5 days.

Table 1

Peptide	Sequence	Concentrations for treatments (µM)
mouse PrP ₁₋₂₈ ^{*,a}	MANLGYWLLALFVTMWTDVGLCKKRPKP-NH ₂	0.1, 0.5, 1 [*] , 2, 5 [*] , 10 [*]
bovine PrP ₁₋₃₀	MVKSKIGSWILVLFVAMWSDVGLCKKRPKP-NH ₂	0.1, 0.5, 1, 2, 5, 10
mouse PrP ₂₃₋₂₈	KKRPKP	0.5, 1, 5, 10, 25, 50
mouse PrP ₁₂₋₂₈ ^a	FVTMWTDVGLCKKRPKP-NH ₂	1, 5, 10
mouse PrP ₂₃₋₅₀	Ac-KKRPKPGGWNTGGSRYPGQGSPGGNRY-P-NH ₂	0.1, 0.5, 1, 2, 5, 10
penetratin	RQIKIWFQNRRMKWKK-NH ₂	0.1, 0.5, 1, 2, 5, 10
penetratin -mPrP ₂₃₋₂₈ ^a	RQIKIWFQNRRMKWKKKKRPKP-NH ₂	1, 5, 10
transportan-10	AGYLLGKINLKALAALAKKIL-NH ₂	0.1, 0.5, 1, 2, 5, 10
transportan-10 -mPrP ₂₃₋₂₈ ^a	AGYLLGKINLKALAALAKKILKKRPKP-NH ₂	1, 5, 10
R ₁₆	RRRRRRRRRRRRRRRR-NH ₂	0.3, 0.6, 3. 6

10

All cell culture reagents and culture plates were purchased from Invitrogen. The peptides penetratin, penetratin-mPrP₂₃₋₂₈, transportan-10 (TP-10), transportan-10-mPrP₂₃₋₂₈, mPrP₁₋₂₈ and 16-mere arginine (R₁₆) were synthesized as described elsewhere (U. Langel, et al. (1992) Int. J. Pept. Protein Res., 39: 516-22). Additional peptides mPrP₁₋₂₈ in L-, D- or D-L configuration, mPrP₂₃₋₂₈, mPrP₂₃₋₅₀ and bPrP₁₋₃₀ were purchased from Neosystem Laboratoire. Primary goat anti-mouse PrP (M-20) sc-1694 antibody and secondary donkey anti-goat Peroxidase-conjugated antibody were from Santa Cruz Botechnology. All other reagents were from Sigma.

20

Cell cultures and RML-infection

GT1-1 cells are murine neuronal hypothalamic cells. Brain ho-
mogenate from Rocky Mountain Laboratories (RML) prion-
5 infected CD-1 mice was kindly provided by Prof. Stanley B.
Prusiner at UCSF, CA. GT1-1 cells were infected with RML
brain homogenate as described previously, at two separate oc-
casions, generating chronically prion-infected cell lines
(ScGT1-1a and ScGT1-1b) (H. Gyllberg, et al. (2006) FEBS
10 Lett, 580: 2603-8; H. Lindegren, et al. (2003) J. Neurosci.
Res., 71: 291-9). A third RML-infected GT1-1 cell culture was
also used (ScGT1-1-RML) as well as GT1-1 cells infected with
brain homogenate from 22L infected mouse (ScGT1-1-22L). In
both these cases, GT1-1 cells were infected according to pro-
15 cedures previously described (H. Gyllberg, et al. (2006)
FEBS Lett, 580: 2603-8; H. Lindegren, et al. (2003) J. Neuro-
sci. Res., 71: 291-9). Cells were cultured as previously de-
scribed (H. Gyllberg, et al. (2006) FEBS Lett, 580: 2603-8).
ScGT1-1a/b cell lines were regularly tested for PrP^{Sc} infec-
20 tion and exhibit proteinase K- (PK) resistance. ScGT1-1a/b
cells can be persistently cured from PrP^{Sc} propagation by
treatment with pentosan polysulfate (PPS).

Peptide treatment of cell cultures

25 0.2x10⁶ GT1-1, ScGT1-1a or ScGT1-1b cells per well were
seeded out in twelve-well petri plates nine days before har-
vest and analysis of prion protein levels. The peptides, as
enlisted in Table 1, were tested for effects on PrP^C levels
30 in GT1-1 cells and effects on PrP^{Sc} levels in ScGT1-1 cells.
In general, peptide treatments were made at final concentra-
tions of 0.1, 0.5, 1.0 and 2.0 µM or if otherwise of concen-
trations as indicated in Table 1, during 3, 5 or 8 days. For
peptides indicated ^a in table 1, treatments were carried out
35 for 5 days only. For penetratin-mPrP₂₃₋₂₈ and transportan-10-
mPrP₂₃₋₂₈, penetratin or transportan respectively were used as
controls. Peptides indicated ^a in table 1, including con-
trols, were tested on ScGT1-1-RML and ScGT-1-22L cell cul-

tures for 5 days in indicated concentrations. Additions of phosphate-buffered saline (PBS) were used for negative controls. For positive controls, cells were treated with 2 μ M of PPS. During 8 days of treatments with peptides at concentrations of 5 or 10 μ M, the medium was changed every 24 hour prior to new peptide addition. This was done in order to avoid toxicity from aggregated peptide not taken up into the cells. ScGT1-1b cells were cultured across several passages (8, 15, 23 and 30 days) in the presence of 0.5 μ M mPrP₁₋₂₈. For all treatments, new peptide was added every 24 hour.

RML infection of GT1-1 cells during peptide treatment

0.2x10⁶ GT1-1 cells per well were seeded out in twelve well petri plates and cultured to reach approximately 80% confluence. RML infection was performed as described above, but in the presence of 10 μ M of either mPrP₁₋₂₈, mPrP₂₃₋₅₀, bPrP₁₋₃₀, penetratin, transportan-10 or 47 μ M of mPrP₂₃₋₂₈. New peptide additions were made every 24 hour during the 3 days of RML-infection. Control cell cultures were exposed to RML infection with addition of PBS instead of peptide. Cultures were passaged for 3 weeks before subsets of each culture were harvested and analyzed for presence of PrP^{Sc} by PK and western blot. Remaining subsets were cultured for additional 2 weeks before harvest and analysis.

Proteinase-K digestion and western blot

Following each respective peptide treatment, cells were extracted in cold lysis buffer (0.5% Triton X-100, 0.5% NaDoc, 150 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.5 at 0 °C). The lysates were cleared by centrifugation for 2 min at 5000xg, and the pellets were removed. Protein concentration was measured by Bradford and protein levels were normalised to 1 mg/ml of protein in all samples. PK-digestion was performed on 500 μ g of protein as described in H. Gyllberg, et al. (2006) FEBS Lett, 580: 2603-8. For PK digestion of heparin conjugated agarose precipitates, 10 μ g of PK was added in a volume of 5

5 μ l lysis buffer without protease inhibitors. Samples were incubated at 37°C for 45 min, and 20 μ l of 4x laemmli sample buffer was added and samples were boiled for 5 min. The samples were separated on a 12% SDS-PAGE followed by transfer to a nitrocellulose membrane and analyzed by western blot with an anti-PrP antibody as described in H. Gyllberg, et al. (2006) FEBS Lett, 580: 2603-8. For immunodetection, enhanced chemiluminescence (ECL) was used.

10 Precipitation of prion protein with heparin-conjugated agarose

2x10⁶ GT1-1 or ScGT1-1b cells were seeded out in 10 cm petri dishes and cultivated for 7 days. Cells were extracted in cold lysis buffer supplemented with protease inhibitors (10 mM phenyl-methanesulphonylfluoride (PMSF), 1 μ g/ml pepstatin, 1 mg/ml aprotinin and 1 mM sodiumorthovanadate). The lysates were cleared and the protein levels were normalized as described above. 500 μ g of protein was used for each sample. 50 μ l of heparin-agarose beads was preincubated for 3 h at room temperature with 200 μ M of mPrP₁₋₂₈ or with lysis buffer as control. In parallel, lysates were preincubated with 100 μ M mPrP₁₋₂₈ or with lysis buffer as control. To preincubated heparin-agarose, lysate was added. To preincubated lysate, 50 μ l of heparin-agarose beads was added. The final volume of all samples was 300 μ l and for samples with mPrP₁₋₂₈, the final concentration of peptide was 67 μ M. The samples were incubated for 3 h at room temperature. The beads were washed 5 times in 1 ml of cold PBS. All fluid was removed and 50 μ l of 2x laemmli buffer was added to the, or the samples were exposed to PK digestion. Samples were analyzed for PrP levels by SDS-PAGE and western blot.

Statistical analysis

35

From the ECL detected films, optical density (OD) measurement was performed using the software Image Gauge V.3.46. OD was measured from western blots of whole cell lysate to receive

values of PrP^C or total PrP, or from PK digested samples to measure levels of PrP^{Sc}. In indicated cases, an alternative way of calculating PrP^{Sc} levels was used; the OD value of the 18 kDa PrP^{Sc} specific band visible on western blots of whole
5 cell lysate was measured and put in relation to the OD of the sample total PrP. PrP^C or PrP^{Sc} levels in peptide treated cell cultures were divided by the levels in untreated controls and expressed in percentage. Statistical analysis of relative protein levels were performed using the software GraphPad
10 Prism V4, and graphs for the respective peptides are shown in supplementary data.

Industrial Applicability

15 Having now fully disclosed the invention, it is clear to the skilled person that it satisfies a long felt need in the art and constitutes a significant contribution thereto. While the invention has been explained and exemplified making use of various materials and methods in the art, a skilled person
20 will appreciate that it is in no way limited thereto, and that the scope of the present invention is as set forth in the appended claims.

Sequence Listing

- MANLGCWMLVLFVATWSDLGLCKKRPKP (SEQ ID NO. 1)
- 5 MANLGYWLLALFVTMTDVGGLCKKRPKP (SEQ ID NO. 2)
- MVKSHIGSWILVLFVAMWSDVGLCKKRPKP (SEQ ID NO. 3)
- MVKSHIGSWILVLFVAMWSDVGLCKKRPKP (SEQ ID NO. 4)
- 10 MVKSHIGSWILVLFVAMWSDVGLCKKRPKP (SEQ ID NO. 5)
- MAHLGYWMLLLFVATWSDVGLCKKRPKP (SEQ ID NO. 6)
- 15 MANLGYWLLALFVTTCTDVGGLCKKRPKP (SEQ ID NO. 7)
- MANLSYWLLALFVATWTDVGGLCKKRPKP (SEQ ID NO. 8)
- MVKSHIGSWILVLFVAMWSDVGLCKKRPKP (SEQ ID NO. 9)
- 20 MVKSHIGSWILVLFVAMWSDVGLCKKRPKP (SEQ ID NO. 10)
- MVKSHIGGWILVLFVAAWSDIGLCKKRPKP (SEQ ID NO. 11)

CLAIMS

1. A prion protein derived cell penetrating peptide (PrP-CPP) with binding affinity for PrP^{Sc}, wherein the PrP-CPP reduces
5 the PrP^{Sc} level in cells.
2. The PrP-CPP according to claim 1, wherein said PrP-CPP is selected from SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID
10 NO. 8, SEQ ID NO. 9, SEQ ID NO. 10 or SEQ ID NO. 11.
3. Use of a PrP-CPP according to claim 1 or 2, for detection of PrP^{Sc} or the PrP^{Sc} level in a cell or a mammal.
- 15 4. Use of a PrP-CPP according to claim 1 or 2, for the manufacture of a diagnostic agent to diagnose or detect a prion disease.
5. The diagnostic agent according to claim 4.
20
6. Use of a PrP-CPP according to claim 1 or 2 as an antigen for the selection, design or production of an immune epitope molecule.
- 25 7. Method for detecting the PrP^{Sc} level in a cell, comprising administering a prion protein derived cell penetrating peptide (PrP-CPP) with binding affinity for PrP^{Sc}.

--MANLGCWMLVLFVATWSDVLGLCKKRRPKP	human
--MANLGYWLLALFVTMWTDVGLCKKRRPKP	mouse
MVKSHIGSWILVLFVAMWSDVGLCKKRRPKP	bovine
MVKSHIGSWILVLFVAMWSDVGLCKKRRPKP	sheep
MVKSHIGSWILVLFVAMWSDVGLCKKRRPKP	goat
--MAHLGYWMLLLFFVATWSDVGLCKKRRPKP	rabbit
--MANLGYWLLALFVTTCTDVGLCKKRRPKP	rat
--MANLSYWLLALFVATWTDVGLCKKRRPKP	Chinese hamster
MVKSHIGSWILVLFVAMWSDVGLCKKRRPKP	cat
MVKSHIGSWILVLFVAMWSDVGLCKKRRPKP	dog
MVKSHIGGWILVLFVAAWSDIGLCKKRRPKP	pig
Signal sequence	NLS-like sequence

Fig. 1

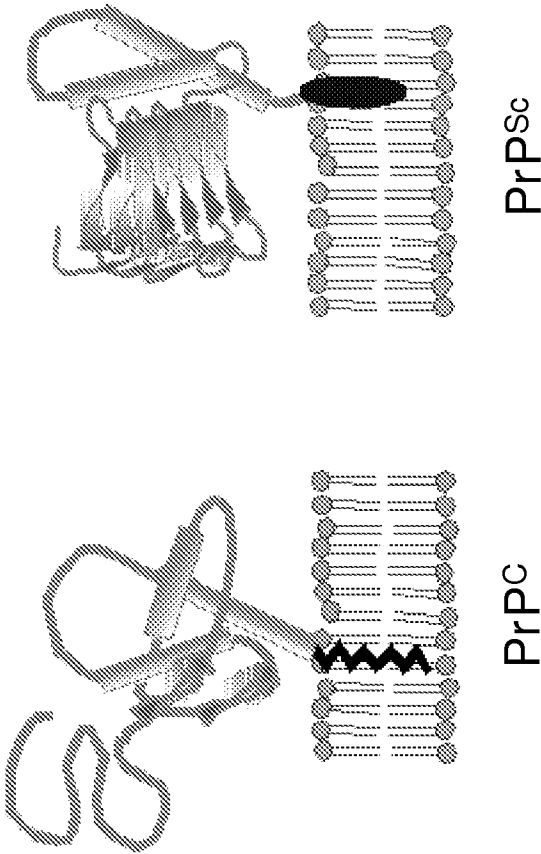


Fig. 2

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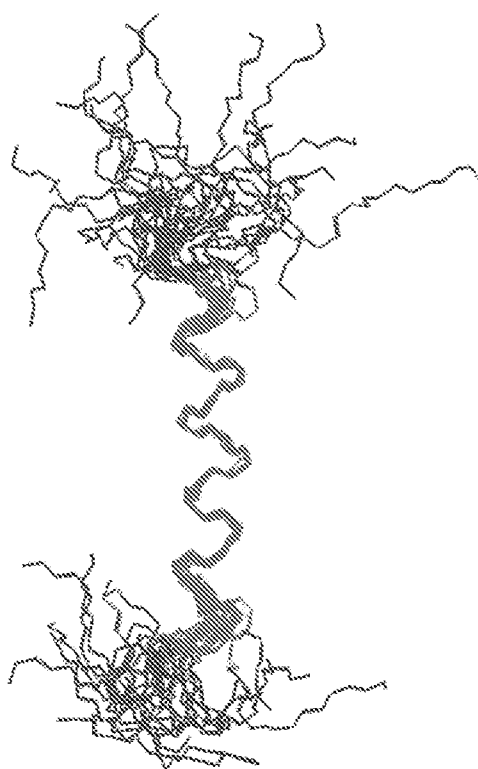
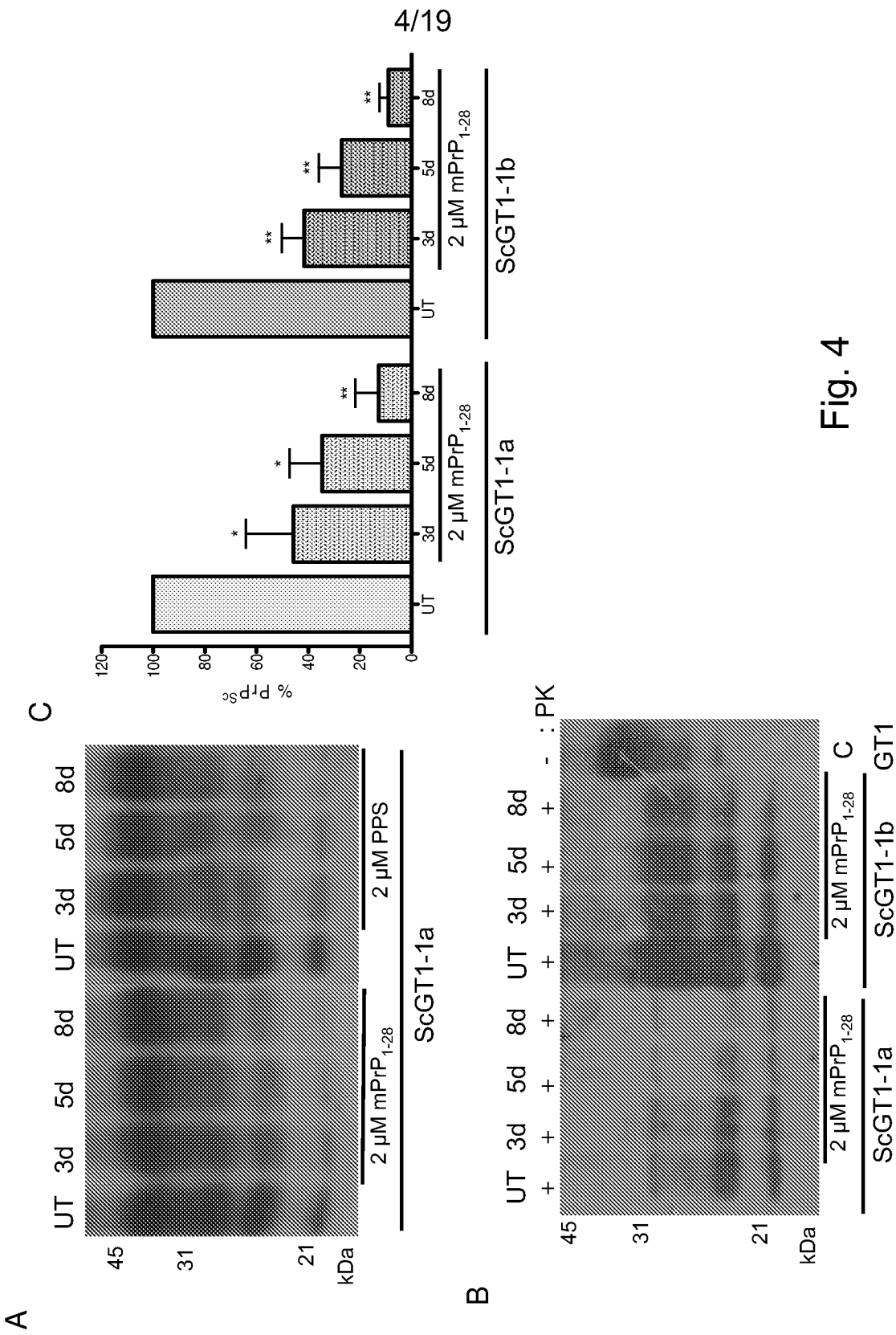


Fig. 3



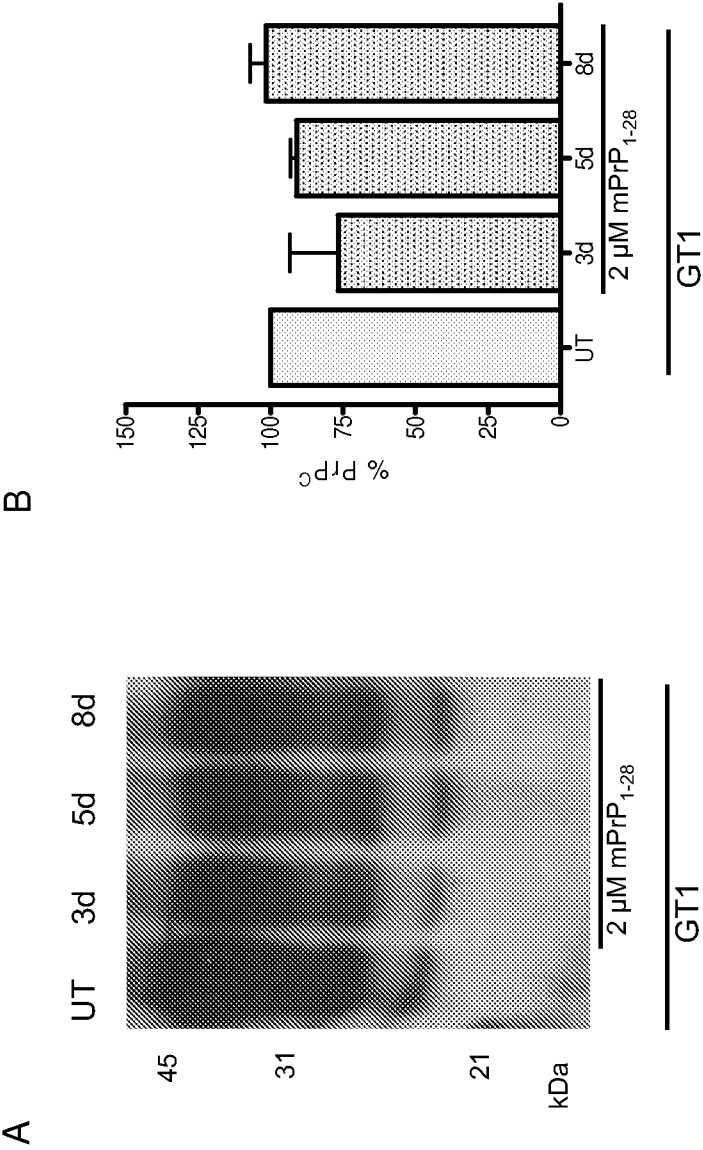


Fig. 5

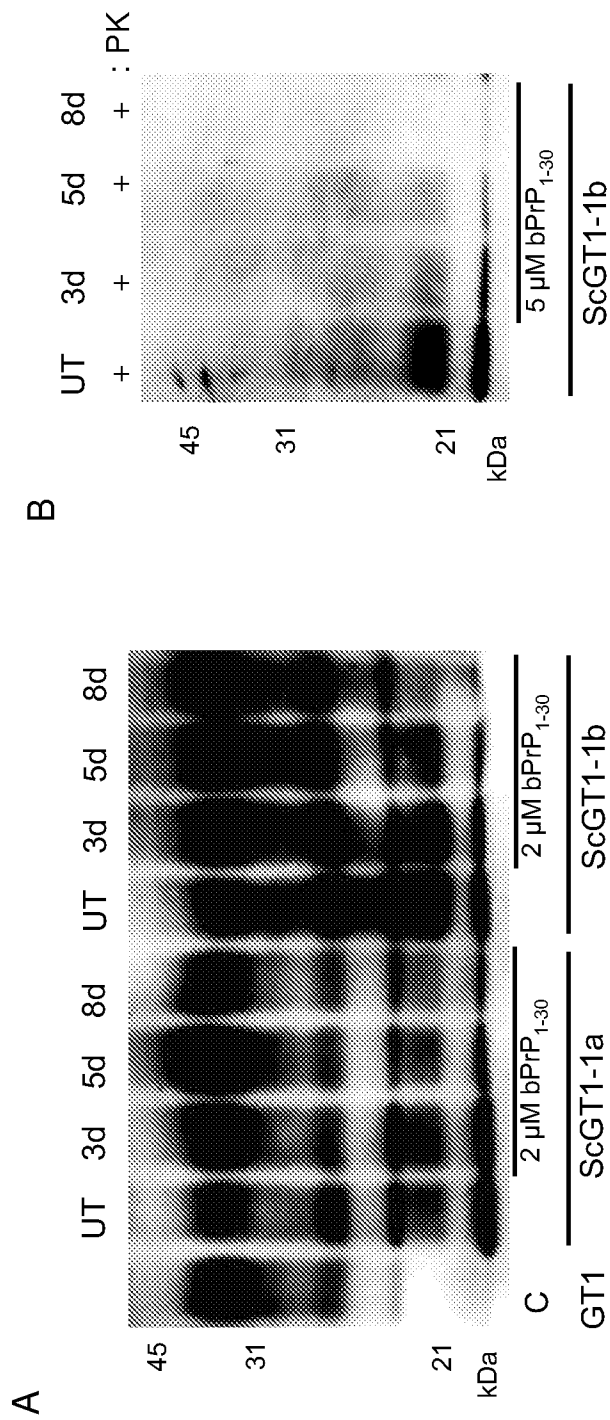


Fig. 6

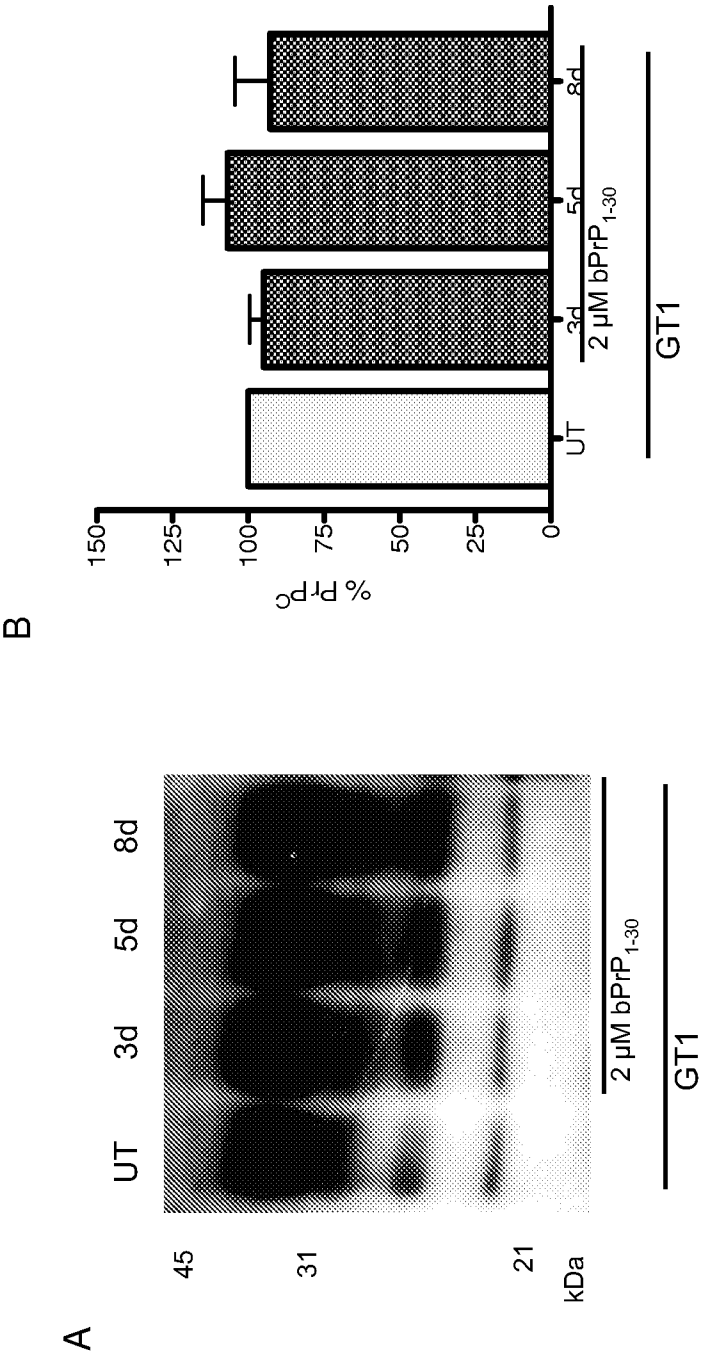


Fig. 7

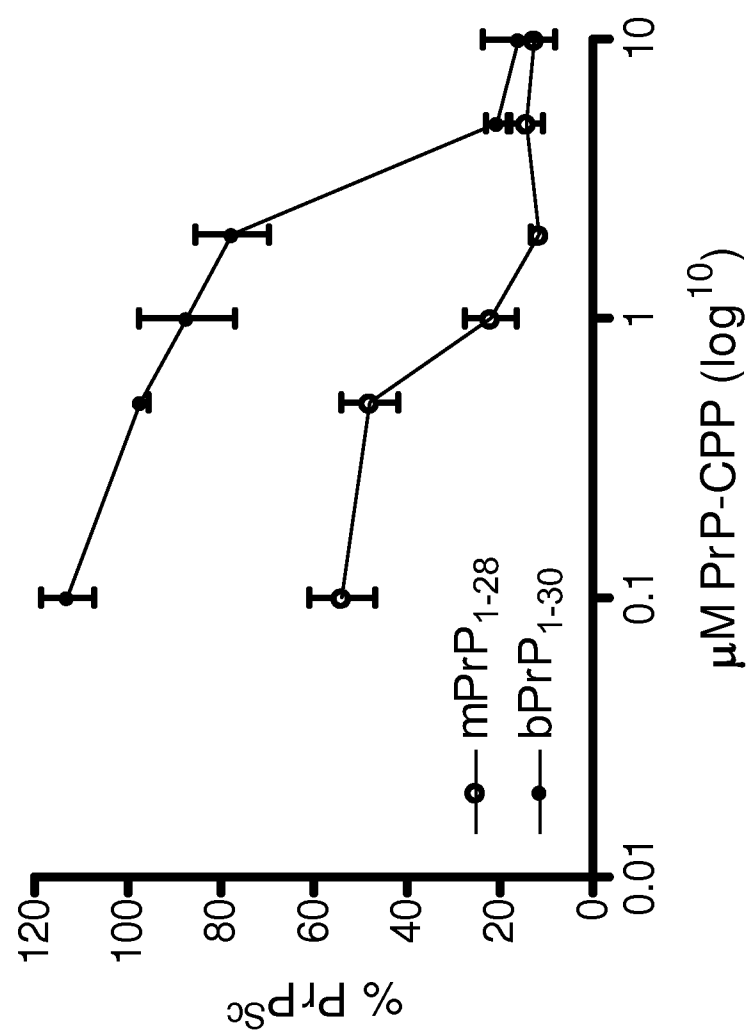


Fig. 8

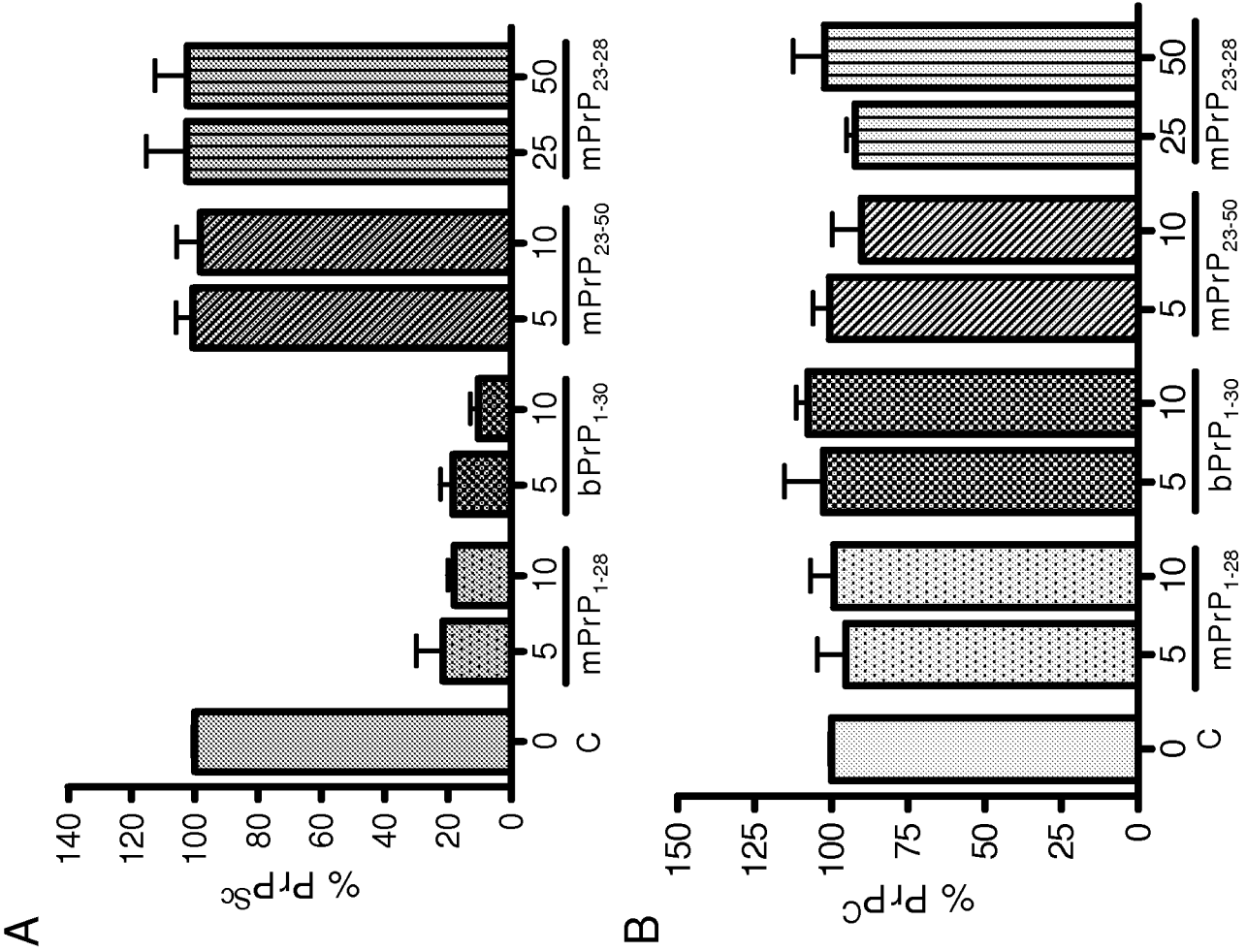


Fig. 9

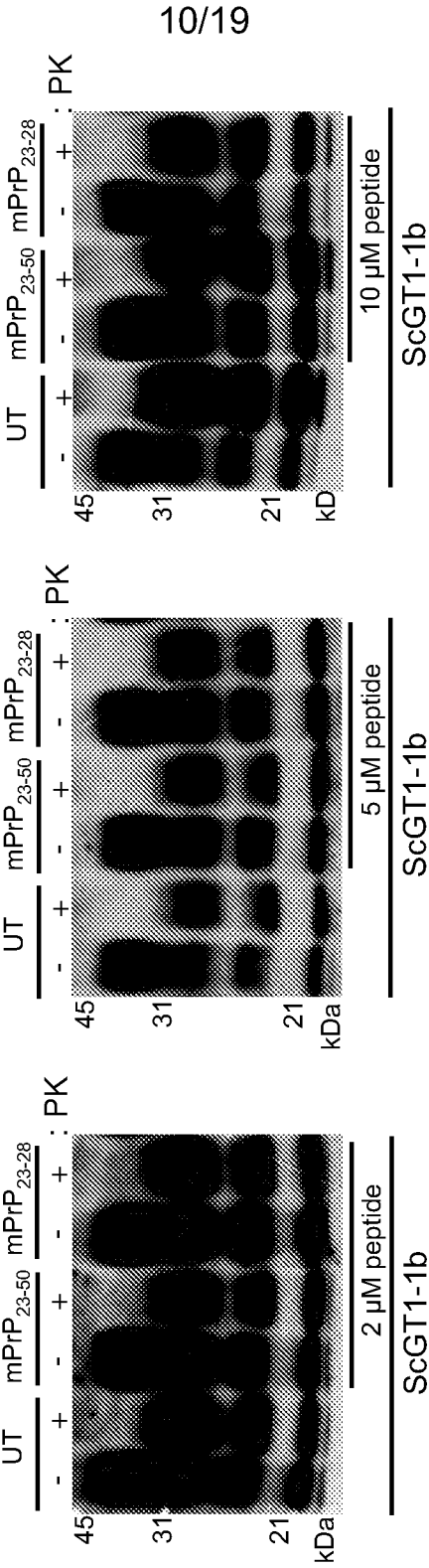


Fig. 10

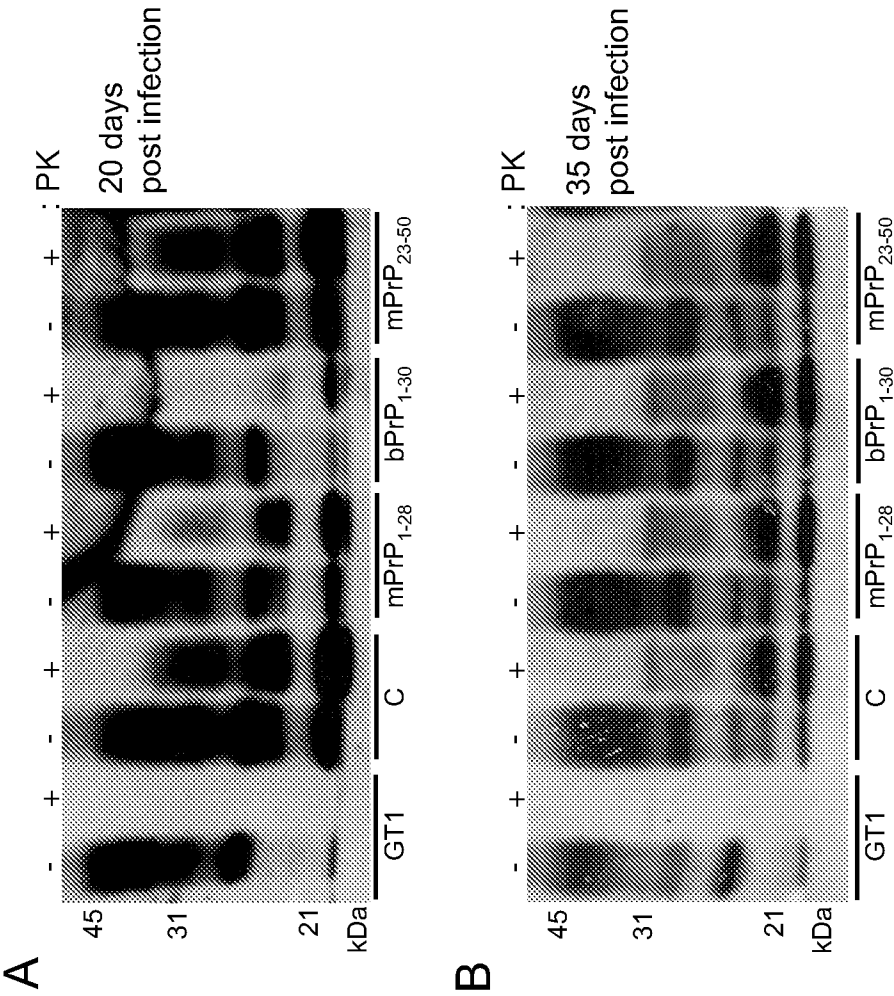


Fig. 11

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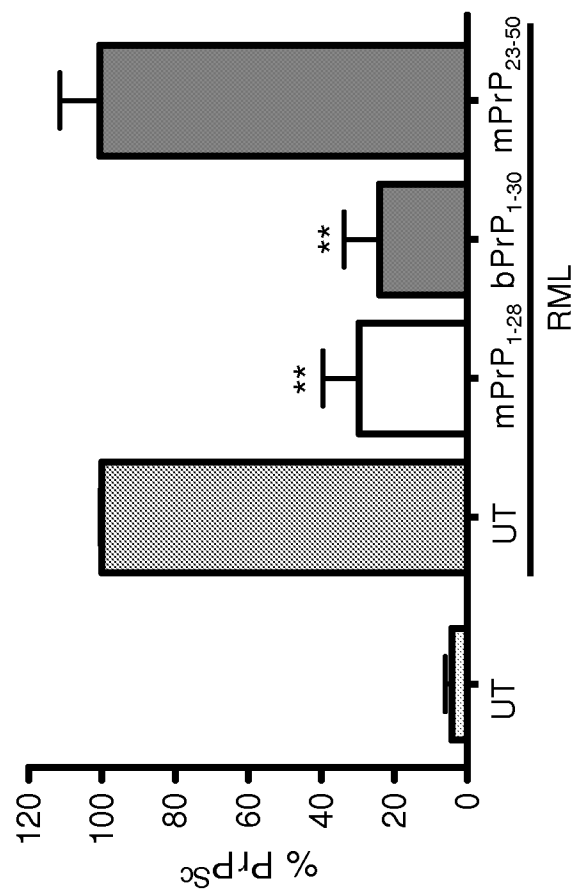


Fig. 12

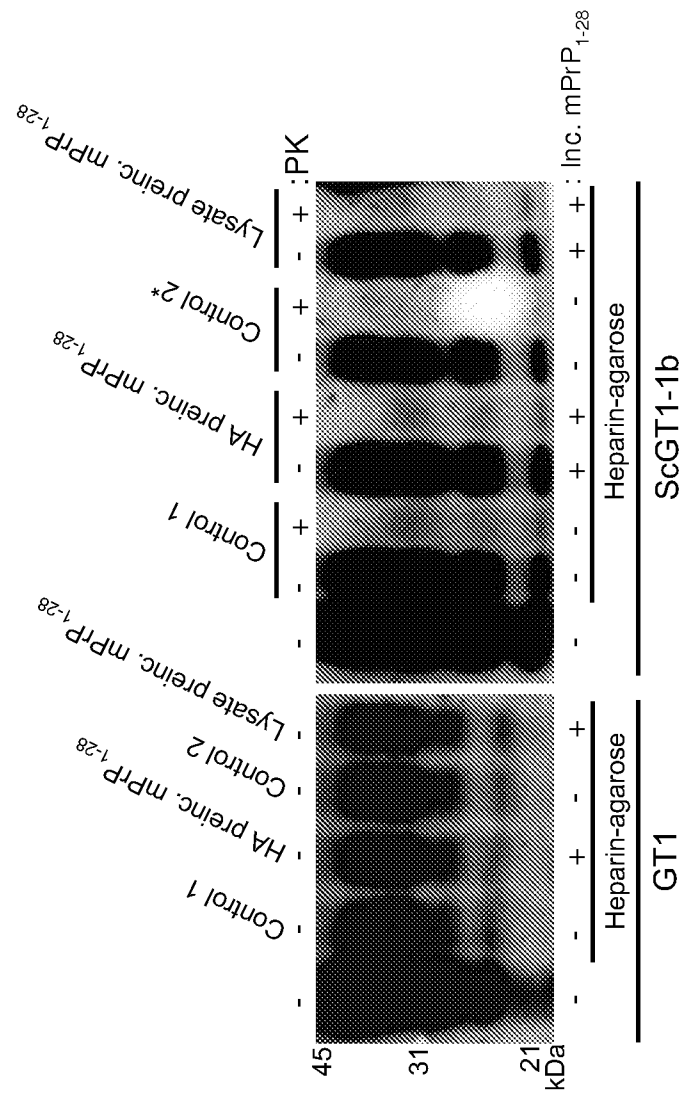
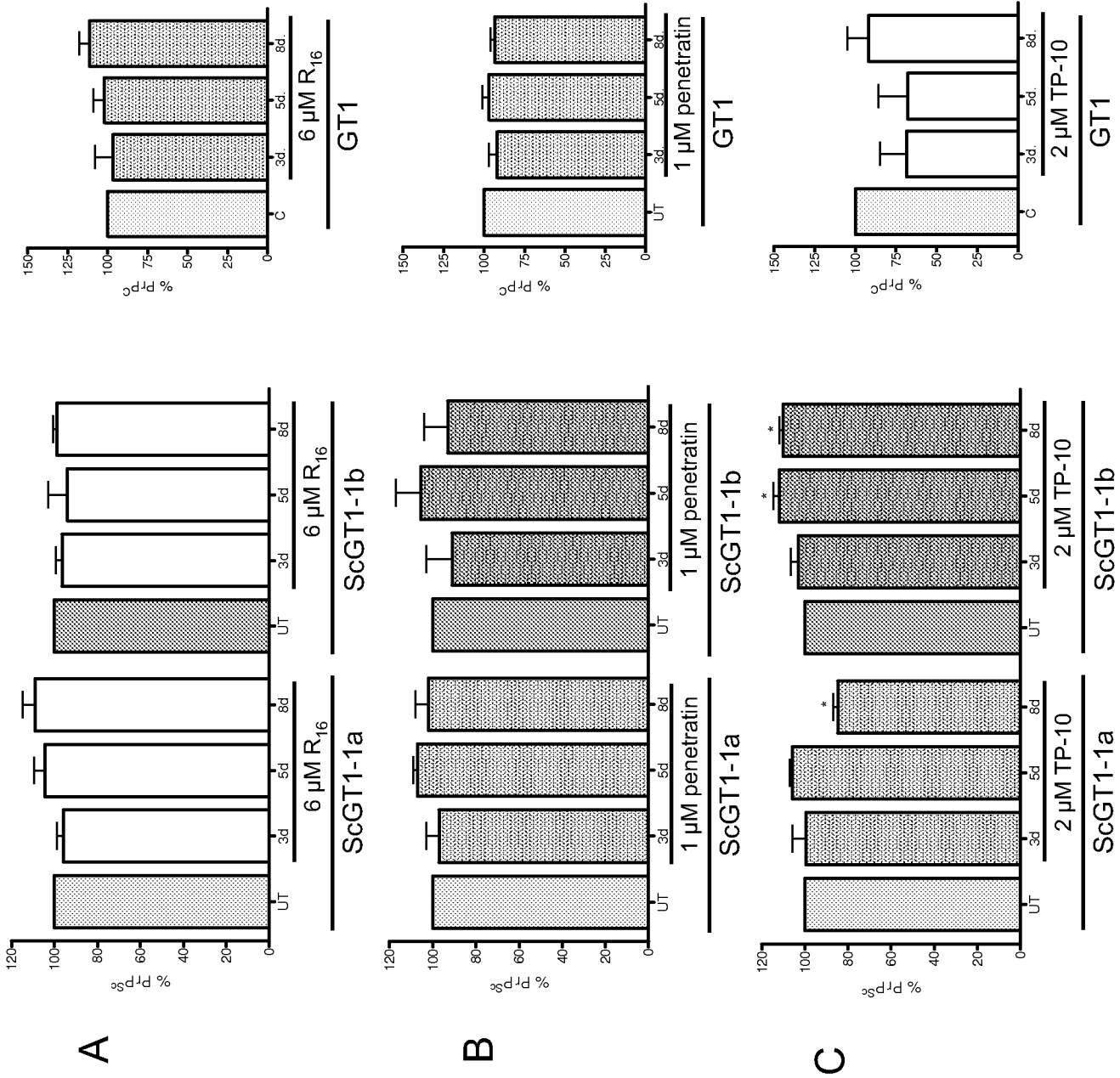


Fig. 13



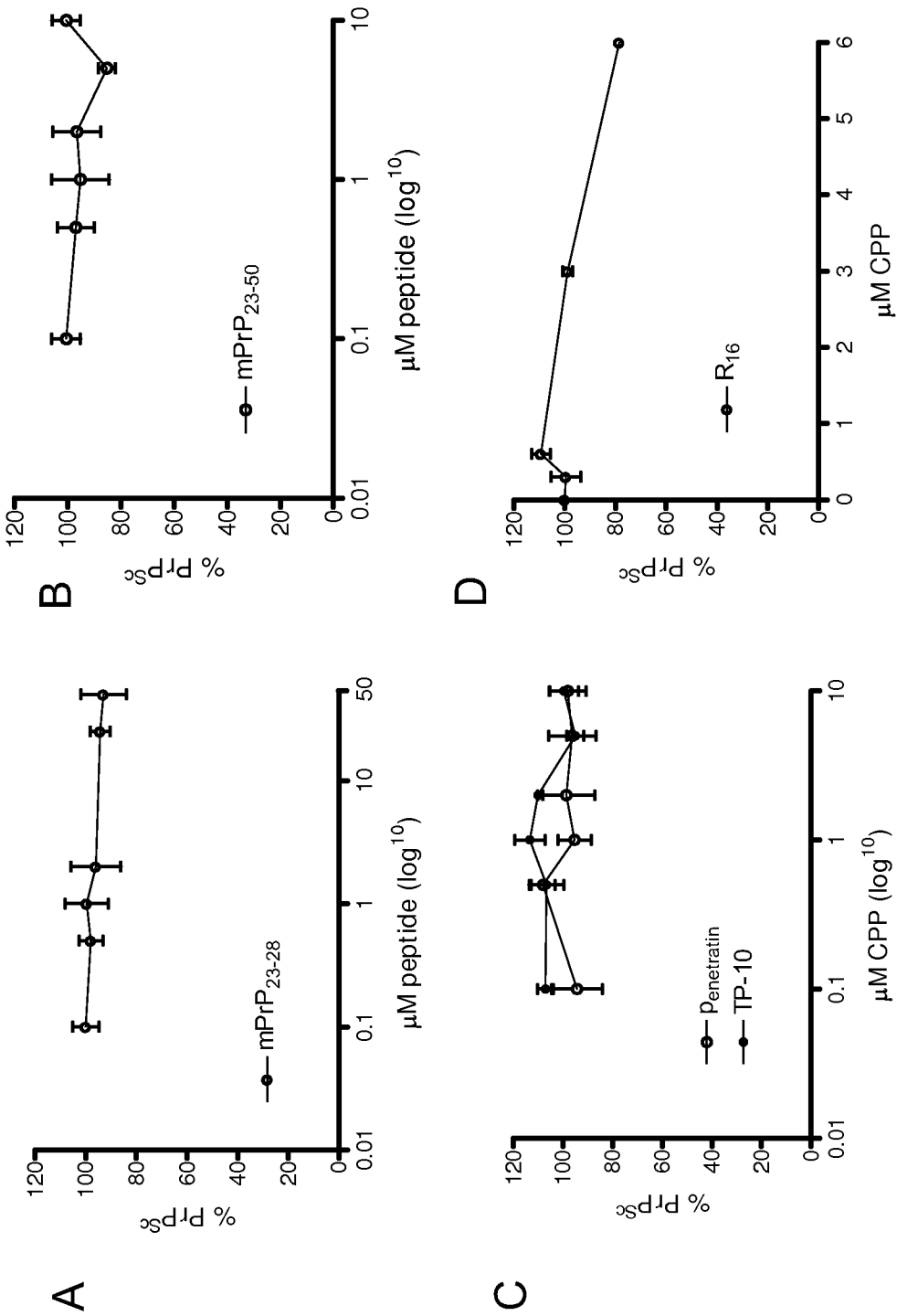


Fig. 15

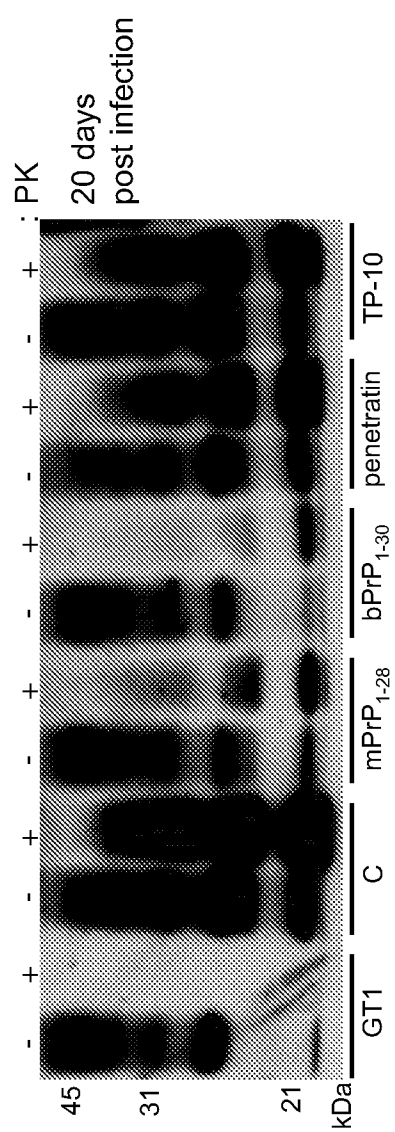


Fig. 16

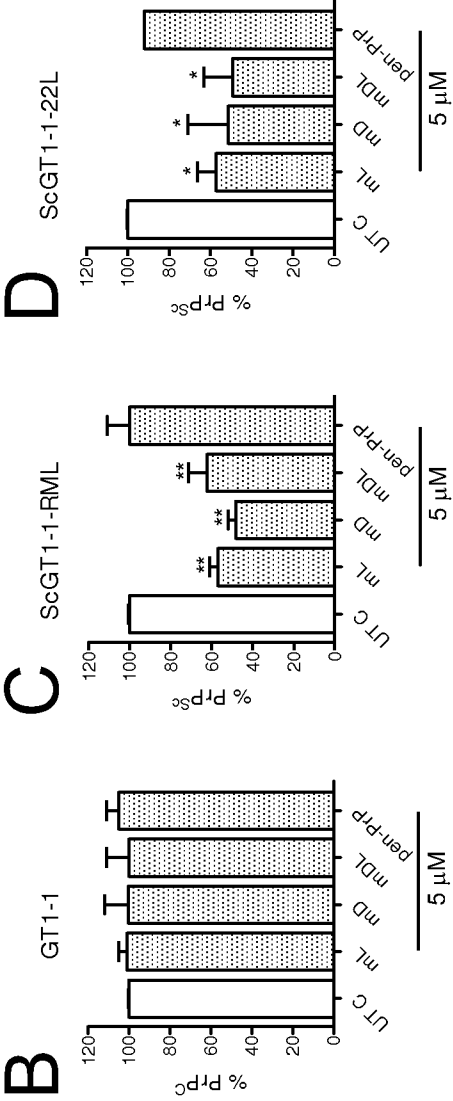
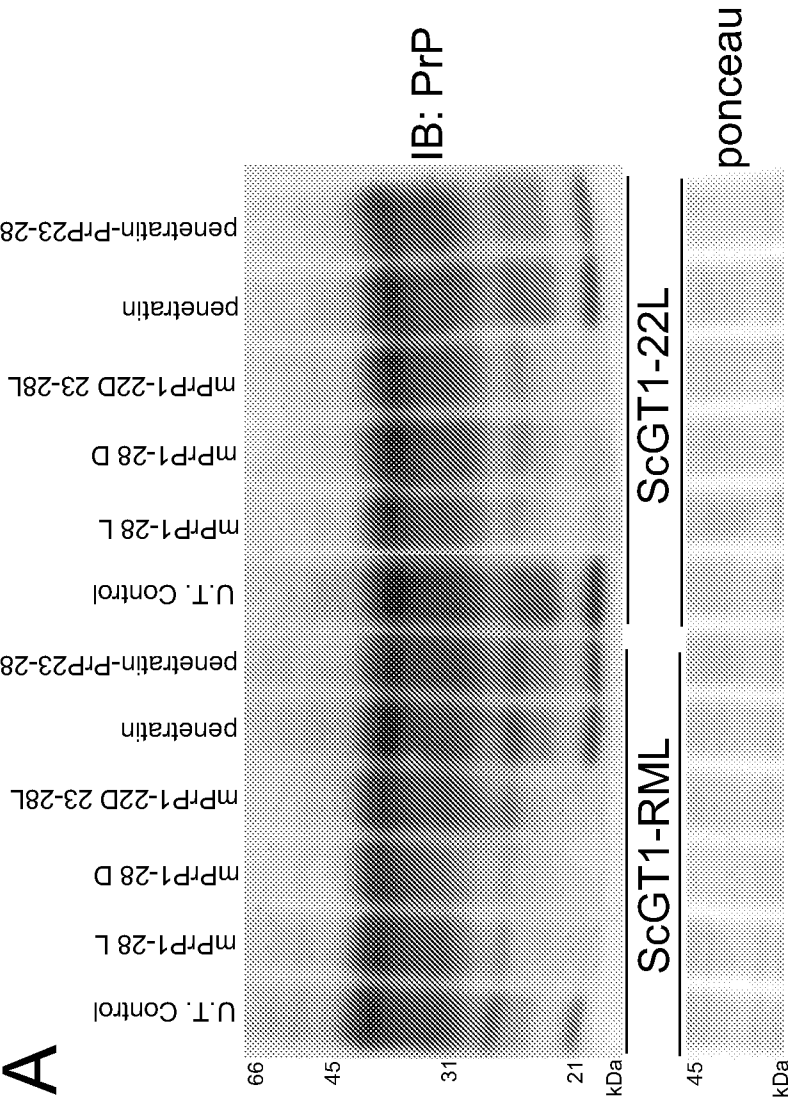


Fig. 17

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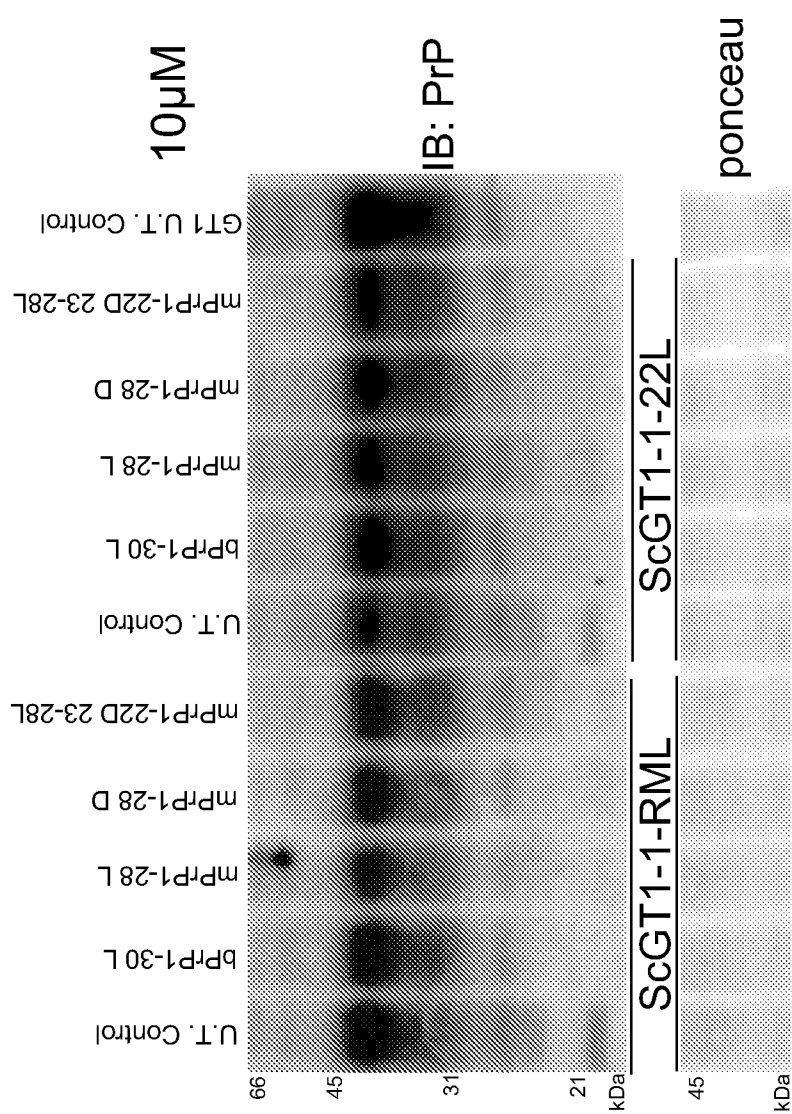


Fig. 18

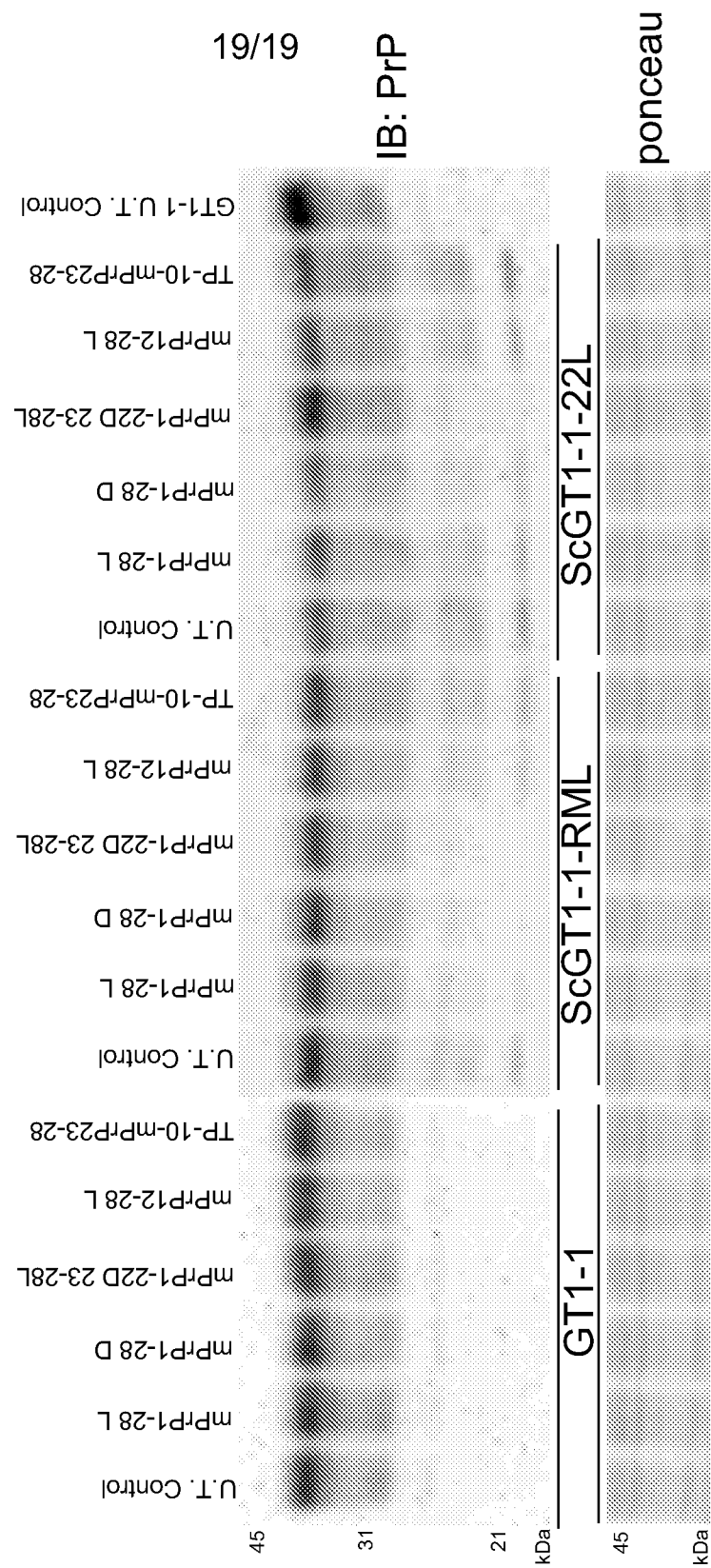


Fig. 19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE2008/051073

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, MEDLINE, EMBASE, BIOSIS, EBI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03106491 A2 (CEPEP AB), 24 December 2003 (24.12.2003), page 33, line 19 - line 25, claims 39,76	1-2
A	--	3-7
X	LUNDBERG P. ET AL: "Cell membrane translocation of the N-terminal (1-28) part of the prion protein", Biochemical and Biophysical Research Communications 2002, Vol. 299, p. 85-90, page 1, column 1, line 1 - page 2, column 1, line 8, abstract	1-2
A	--	3-7

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

24 October 2008

Date of mailing of the international search report

29-10-2008

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE2008/051073

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 20050118645 A1 (MICHELITSCH ET AL), 2 June 2005 (02.06.2005), claims 1-3,9,22,33, abstract, paragraphs [0001],[0045],[01115]	1,3-7
A	--	2
A	WO 2006036410 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 6 April 2006 (06.04.2006), paragraphs [0025]-[0031]	1-7
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X	LAU ANTHONY L. ET AL: "Characterization of prion protein (PrP)-derived peptides that discriminate full-length PrPSc from PrPC", PNAS July 10, 2007, Vol. 104, No. 28, p. 11551-11556 Entire document	1,3-7
A	--	2
X	MAGZOUN MAZIN ET AL: "N-terminal peptides from unprocessed prion proteins enter cells by macropinocytosis", Biochemical and Biophysical Research Communications 2006, Vol. 348, p. 379-385, Introduction, abstract	1
A	-- -----	2-7

International patent classification (IPC)

G01N 33/68 (2006.01)
C07K 14/47 (2006.01)
C07K 16/28 (2006.01)
A61K 38/17 (2006.01)
A61P 25/00 (2006.01)

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Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/08/2008

International application No.

PCT/SE2008/051073

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				RU	2006107565	A	27/08/2006
				WO	2005016127	A	24/02/2005

WO	2006036410	A2	06/04/2006	US	20080027025	A	31/01/2008
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