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Piperazine derivatives inhibit PrP/PrP^{res} propagation *in vitro* and *in vivo*

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ABSTRACT

Prion diseases are fatal neurodegenerative disorders, which are not curable and no effective treatment exists so far. The major neuropathological change in diseased brains is the conversion of the normal cellular form of the prion protein PrPc^{C} into a disease-associated isoform PrP^{Sc}. PrP^{Sc} accumulates into multimeres and fibrillar aggregates, which leads to the formation of amyloid plaques. Increasing evidence indicates a fundamental role of PrP^{Sc} species and its aggregation in the pathogenesis of prion diseases, which initiates the pathological cascade and leads to neurodegeneration accompanied by spongiform changes. In search of compounds that have the potential to interfere with PrP^{Sc} formation and propagation, we used a cell based assay for the screening of potential aggregation inhibitors. The assay deals with a permanently prion infected cell line that was adapted for a high-throughput screening of a compound library composed of 10,000 compounds (DIVERset 2, ChemBridge).

We could detect six different classes of highly potent inhibitors of PrP^{Sc} propagation *in vitro* and identified piperazine derivatives as a new inhibitory lead structure, which increased incubation time of scrapie infected mice.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are infectious, neurodegenerative disorders in humans and animals and include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans. The appearance of a novel form of human prion disease, variant CJD (vCJD), which has been linked to BSE, intensified the research activities for the discovery of new therapeutic agents.

TSEs are characterized by the accumulation of an abnormal isoform of the prion protein (PrP^{Sc}), derived from cellular prion protein (PrP^{C}). According to the "protein-only" hypothesis, PrP^{Sc} is the principal constituent of the transmissible agent, even though the exact nature of the infectious agent remains unclear. The host-encoded, cellular prion protein (PrP^{C}), however, is essential for disease development and converts into a fibrillogenic and partially proteinase K (PK) resistant isoform, which has a high content of β-sheet structure [1]. Up to now, no treatment is available, that stops the disease process or prevents neuronal degeneration. Therefore, there is a strong interest to identify lead compounds that inhibitthe PrP conversion and/or PrP^{Sc} aggregation and might be developed into therapeutic drugs.

The discovery of new anti-prion compounds was expedited by novel *in vitro* systems such as fluorescence correlation spectroscopy [2], cell-free conversion assays [3] and cell culture models [4]. Compound screening yielded new inhibitory compounds like benzohydrazides [2], 2-aminothiazoles [5], di-phenyl-pyrazoles [6,7] and benzothiazoles [8]. Although many of these compounds were also effective when tested in mouse models for prion diseases, only few have made their way into human trials. Case reports were published for acyclovir [9,10], quinacrine [11] and flurpitrine [12]. There use lead to a more general but only modest clinical improvement but no significant delay of disease progression or survival time could were observed. Therefore further efforts should be made to find better therapies.

Here we present a high-throughput approach by screening a compound library of 10,000 compounds (DIVERset 2, ChemBridge) using a cell-based assay. We detected six different classes of

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inhibitors of PrP^{Sc} propagation *in vitro* and identified piperazine derivatives a new lead structure, which increased the incubation time of scrapie infected mice significantly after intraperitoneal application.

2. Materials and methods

2.1. Chemicals

Compounds purchased from ChemBridge Corporation (San Diego, USA) and courtesy provided by A. Giese and H. Kretzschmar (München), are supplied for *in vitro* testing as 1–2 mM solutions in dimethyl sulfoxide (DMSO). In terms of *in vivo* testing compounds were dissolved in 100% DMSO or were suspended in physiological saline (0.9% NaCl) just before use. Compounds purchased from Setec Astronomy GmbH or Sigma–Aldrich were dissolved in 100% pure water or in physiological saline (0.9% NaCl) just before use.

2.2. Testing for inhibition of PrP^{Sc} accumulation in a cell-based dot blot model

Approximately 20,000 scrapie mouse brain (SMB) cells infected with the Rocky Mountain Laboratory scrapie strain (RML) were suspended in 100 µl of medium and were per well seeded into a Costar 3599 flat-bottom 96-well plate (Corning Inc., Corning, NY) prior to the addition of the test compounds. Test compound solutions (1–2 mM) were diluted in DMSO prior to being added to the cell medium. One microliter of each solution was added to the cell medium. After incubation at 35 °C in a CO_2incubator for 3 days, the cultures were lysed and analyzed for PrP^{res} expression.

Prior to cell lysis, the cultures were controlled for toxicity of the compounds and density of the cell culture in comparison to the controls by light microscopy. After removal of the cell medium, 100 µl of lysis puffer (50 mM Tris–HCl [ph 8.0], 150 mM NaCl; 0.5% [wt/vol] deoxycholic acid sodium salt [DOC]; 0.5% [vol/vol] Triton X-100) was added to each well for 5 min at room temperature. Using a dot blot apparatus (Sigma–Aldrich), the cell lysate was transferred to an activated polyvinylidenedifluoride (PVDF) membrane (Immobilon-P; Milipore) under vacuum pressure and fixed to the membrane for 1 h at 37 °C. After that the membrane was incubated simultaneously in lysis puffer and treated with (PK solution (final concentration, 25 µg/ml) for 90 min at 37 °C. The membrane was then washed twice with pure water. Denaturation solution (3 M guanidiniumthiocyanat, 0.1 M Tris–HCl [ph 8.0],) was added for 10 min at room temperature. Afterwards, the membrane was washed 5 times with pure water. After the denaturation the membrane was blocked with 5% (wt/vol) non-fat milk containing 0.1% (vol/vol) Tween20 (Sigma) in phosphate-buffered saline (PBST-milk) for 60 min. The polyclonal antibody (pab) Ra10 [13] was incubated in the blocking solution with the membrane for 60 min. After rinsing in PBST for 3 times, the membrane was incubated in 0.2 µg of horseradish peroxidise-conjugated, anti-rabbit IgG (Promega)/ml in PBST-milk at room temperature for 1 h. After additional rinsing in PBST, the bound antibodies were detected by using a chemiluminescence reagent system (ECL, Amersham) and were visualized directly in an image analysis system (Versa Doc, Quantity One, Bio-Rad, Munich, Germany). Together with each dilution series of the compounds, untreated control wells and wells that were treated with suramin, a known PrP^{Sc} inhibitor in RML infected mouse neuroblastoma cells (ScN_2a) and SMB cells, were analysed [14].

In addition, we determined the half maximal inhibitory concentration (50% inhibitory concentration [IC_{50}]) values for each of the effective compounds from dose–response curves after the application of 10-fold dilutions (from 20 to 2 µM and down to 0.2 µM) of each compound to the corresponding cell line.

2.3. Testing for PrP^{C} expression in uninfected mouse neuroblastoma cells (N_2a)

Approximately 20,000 N_2a cells were seeded into 96-well-plates. Further processing including compound application, cell lysis and PrP^{C} detection was carried out as described earlier.

2.4. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] test (Sigma)

About 20,000 N_2a cells were added to each well of a 96-wellplate. The cells were allowed to settle before test compounds were added to a final concentration of 20 µM and incubated for 3 days. After that, the MTT-test was conducted according to the manufacturer's instructions. In this test the tetrazolium salt was converted into blue insoluble formazan dye crystals, which had to be dissolved by a suitable extraction mixture. The formazan absorption was spectro-photometrical quantified (570 nm test wavelength; 630 nm reference wavelength).

2.5. Cell-free conversion assay

This assay was carried out according to the protocol established by Eiden et al. [15] and Kupfer et al. [16]. In brief, cell-free conversion was carried out by mixing bacterial recombinant murine 3F4-tagged prion protein (rPrP) with purified PrP^{Sc} seeds (mouse passaged scrapie strain Me7) in an appropriate conversion buffer. After digestion with PK for 1 h at 37 °C (end dilution, 30 µg/ml), newly generated PrP^{res} fragments were detected by immunoblotting and incubation with monoclonal antibody (mab) 3F4 [17]. After treatment of the membranes with stripping buffer, PrP^{Sc} aggregates were detected by using pab Ra10. Compounds were added to the conversion reaction to a final concentration of 100 µM and analysed with regard to inhibition of PrP^{res} formation and dissolution of PrP^{Sc} aggregates.

2.6. Proteasome-Glo cell-based assay (promega)

About 20,000 N_2a cells were added to each well of a 96-well-plate. The cells were allowed to settle before test compounds were added to a final concentration of 20 µM and incubated for 3 days. After that, the assay was conducted according to the manufacturer's instructions.

Measurement of the activity of the proteasome complex in cultured N_2a cells provides a luminogenic proteasome substrate, Suc-LLVY-amnioluciferin, in a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. Addition of the Proteasome-Glo cell-based assay reagent in an add-mix-measure format results in proteasome cleavage of the substrate and rapid generation of a luminescent signal produced by the luciferase reaction.

2.7. Western blotting

PrP^{Sc} accumulation was examined by Western blotting using phosphotungstic acid (PTA) precipitations, which were carried out according to the protocol established by Wadsworth et al. and Gretzschel et al. [18] with some modifications [19].

10% (wt/vol) brain homogenatesfor all samples were prepared in 0.42 mM sucrose solution containing 0.5% deoxycholic acid sodium salt (DOC) and 0.5% Nonidet P-40 (NP 40) using a Ribolyser (Hybaid, Heidelberg, Germany). Aliquots were adjusted with PK

(Boehringer Mannheim) to a final concentration of 50 μg of PK/ml, followed by incubation at 55 °C for 60 min. Digestion was stopped by addition of Pefabloc (Roche, Mannheim, Germany) and heated to 95 °C for 5 min. Subsequently, PTA was added to the samples (final concentration, 0.3% [wt/vol]) which were incubated at 37 °C for 60 min with constant agitation before centrifugation at 13,300 rpm for 30 min at room temperature. The pellets were resuspended in loading buffer [1% (wt/vol) sodium dodecyl sulphate (SDS), 25 mM Tris–HCl (pH 7.4), 0.5% mercaptoethanol and 0.001% bromophenol blue], heated for 5 min at 95°C and loaded on 16% Tris–Polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride membrane (Millipore) and the membranes were blocked for 1 h in PBST-milk. PrP^{Sc} was detected using mab SAF70 (Spibio) at a concentration of 0.03 μg/ml which was incubated on the membranes for 1 h at room temperature. The membranes were then washed 3 times in PBST and then incubated at a 0.15 μg/ml concentration of alkaline-phosphatase-conjugated anti-mouse immunoglobulin (Dianova) in PBST for 1 h at room temperature. The membranes were finally washed 3 times in PBST and the bound antibodies were detected using the chemiluminescence substrate CDP Star (Tropix) and signals were visualized directly in an image analysis system (Versa Doc, Quantity One, Bio-Rad, Munich, Germany).

2.8. Propagation of scrapie strains

Wild-type C57BL/6 mice were inoculated intracerebrally (i.c.) with 30 μl aliquot of 1% homogenate of RML. Mouse health status was inspected daily and their body weights were recorded weekly. After the onset of TSE-associated clinical symptoms (abnormal tail tonus, hind limb parenthesis or weight loss), the animals were euthanized. The incubation time was calculated as the time between inoculation and death. The brains were removed and one half of each brain was stored at −20 °C and the other half was fixed in 4% natural buffered formalin. Mice that died from other causes than scrapie were excluded from the data.

2.9. Intraperitoneal injection

The daily treatment with 100 μg (5 mg/kg/day) of piperazine derivative (PD) 1–4 dissolved in 100% DMSO started 90 days after the i.c. inoculation of C57BL/6 mice with RML and was administered intraperitoneal (i.p.) over a period of 20 days (Primary efficiency test). We treated 5 mice per compound. As a control, 6 inoculated mice were injected with pure DMSO and were used as dissolving solution control (DMSO-control). In addition 6 mice served as control without injection.

2.10. Statistical analysis

Survival times were analyzed by Kaplan–Meier Survival analysis using a log-rank test to compare the curves. The statistical analysis was performed using SigmaBlot statistical software (San Jose, CA). The survival times are expressed as means ± the standard deviations.

Table 1A
High potent inhibitory compounds from diverset 2 library.

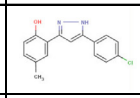
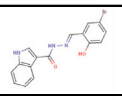
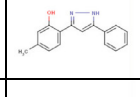
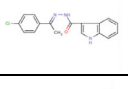
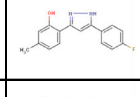
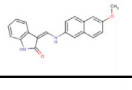
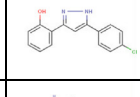
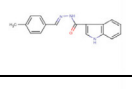
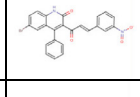
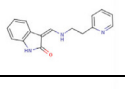
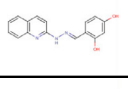
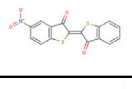
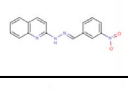
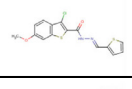
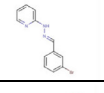
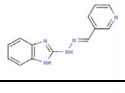
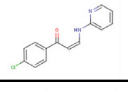
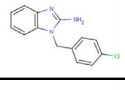
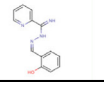
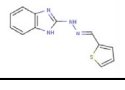
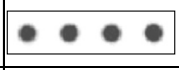
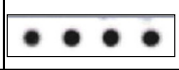
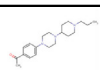
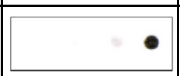
lead compound	Structure	name	IC50 [μM]	lead compound	Structure	name	IC50 [μM]
Diphenylpyrazole		2-[5-(4-chlorophenyl)-1H-pyrazol-3-yl]-4-methylphenol	0,2	Indole		N'-(5-bromo-2-hydroxybenzylidene)-1H-indole-3-carbohydrazide	0,2
		5-methyl-2-(5-phenyl-1H-pyrazol-3-yl)phenol	0,2			N'-[1-(4-chlorophenyl)ethylidene]-1H-indole-3-carbohydrazide	0,2
		2-[5-(4-fluorophenyl)-1H-pyrazol-3-yl]-5-methylphenol	0,1			3-[[(6-methoxy-2-naphthyl)amino]methylene}-1,3-dihydro-2H-indol-2-one	0,2
		2-[5-(4-chlorophenyl)-1H-pyrazol-3-yl]phenol	0,1			N'-(4-methylbenzylidene)-1H-indole-3-carbohydrazide	0,2
Quinolone		3-nitrobenzaldehyde 2-quinolinylhydrazone	1,8			3-{[(4-fluorophenyl)amino]methylene}-1,3-dihydro-2H-indol-2-one	0,1
		6-bromo-3-[3-(3-nitrophenyl)acryloyl]-4-phenyl-2(1H)-quinolinone	1,5	Benzothiophen		5-nitro-3H,3'H-2,2'-bi-1-benzothiophene-3,3'-dione	0,5
		2,4-dihydroxybenzaldehyde 2-quinolinylhydrazone	0,2			3-chloro-6-methoxy-N'-(2-thienylmethylene)-1-benzothiophene-2-carbohydrazide	0,3
Pyridine		3-bromobenzaldehyde 2-pyridinylhydrazone	0,2	Benzimidazole		nicotinaldehyde 1H-benzimidazol-2-ylhydrazone	0,5
		1-(4-chlorophenyl)-3-(2-pyridinylamino)-2-propen-1-one	0,2			1-(4-chlorobenzyl)-1H-benzimidazol-2-amine	0,2
		N'-(2-hydroxybenzylidene)-2-pyridinecarboximidohydrazide	0,2			2-thiophenecarbaldehyde 1H-benzimidazol-2-ylhydrazone	0,2

Table 1B
Inhibitory piparazine compounds from diverset 2 library.

Designation	compound	structure	Dot blot				IC_{50}[μM][a]	MTT-Test	Inhibition of PrP Expression in N2a cells				Inhibition in cell-free assay	Influence on proteasome activity
			20 μM	2,0 μM	0,2 μM	control			20 μM	2,0 μM	0,2 μM	control		
PD1	1-{4-[4-(1-benzyl-4-piperidinyl)-1-piperazinyl]phenyl} ethanone						0,1	non toxic					no effect	no influence
PD2	1-(3-chlorophenyl)-4-(2,3-dihydro-1H-inden-2-yl) piperazine						0,2	non toxic					no effect	no influence
PD3	1-(2,3-dihydro-1H-inden-2-yl)-4-(2-pyridinyl)piperazine						0,4	non toxic					no effect	no influence
PD4	1-{4-[4-(1-propyl-4-piperidinyl)-1-piperazinyl]phenyl} ethanone						0,2	non toxic					no effect	no influence

3. Results

3.1. In vitro analyses

The screening of compounds was carried out using cell-based assays using SMB cells and sequentially diluted compounds. The compounds were tested in a first screen at a final dilution of 20 μM and inhibitory substances retested in a secondary round at a final dilution of 20 μM. Starting with 10,000 compounds, 354 potential inhibitors were identified in the first screening round and 52 of them remained after the secondary screen (Table 1A). These compounds belonged to six structural classes or lead compounds: diphenylpyrazoles, benzimidazoles, indoles, benzothiophenes, qinolines and piperazine derivatives (Table 1A). Four piperazine derivatives (PD 1–4) resembled a new class of PrP^{res} conversion/amplification inhibitors and therefore were further analysed *in vitro* and *in vivo*. Structures, dot blot results and IC_{50} values of these compounds are summarized in Table 1B.

1-{4-[4-(1-Benzyl-4-piperidinyl)-1-piperazinyl]phenyl}ethanone (PD1) exhibited an IC_{50} value of 0.1 μM in SMB cells whereas 1-(3-chlorophenyl)-4-(2,3-dihydro-1H-inden-2-yl) piperazine (PD 2) showed an IC_{50} of 0.2 μM. IC_{50} values of 1-(2,3-dihydro-1H-inden-2-yl)-4-(2-pyridinyl)piperazine (PD3) and of 1-{4-[4-(1-propyl-4-piperidinyl)-1-piperazinyl]phenyl}ethanone (PD 4) were 0.4 and 0.2 μM in SMB cells, respectively (Table 1B). Moreover, all inhibitory substances worked *in vitro* without causing cytotoxic effects, as it was shown in the MTT test (Table 1B).

To further study potential molecular inhibitory mechanism, we used a cell-free conversion assay, in which compounds were added to the samples at a concentration of 100 μM (Fig. 1). Newly formed PrP^{res} fragments were detected using a specific antibody (Fig. 1A). In this *in vitro* assay, the PrP^{res} formation was not affected by PD 1–4 (Fig. 1A, lanes 5–8) compared to the untreated control (Fig. 1A, lane 4). Moreover, none of the compounds interfered directly with the added PrP^{Sc} seeds and dissolved them (Fig. 1B, lanes 5–8). No effects were observed for the proteasome activities and the PrP^{C} expression levels in N_2a cells (Table 1B).

3.2. In vivo testing

The four piperazine derivates that displayed a strong inhibitory effect on SMB cells were eventually tested for their efficacy by mouse bioassay using C57BL/6 wild type mice that were challenged i.c. with the RML scrapie strain. PD 1–4 were administered by daily i.p. injection over a period of 20 days, starting 90 days after the i.c. infection. The results of this experimental approach are summarized in Fig. 2A. Untreated control mice displayed a mean incubation time of 144 ± 1 days, which was similar to that of the DMSO control mice (144 ± 1 days). All piperazine derivatives prolonged the mean incubation time of the scrapie infected mice in comparison to the control groups (Fig. 2B). In the case of PD 1 as well as PD 2 the mean incubation time was extended to 157 ± 3 days and 157 ± 4 days, respectively. Treatment with PD 3 significantly increased the incubation time by 23 days (167 ± 1 days) and PD 4 by 18 days (from 144 ± 1 days to 162 ± 5 days). The statistical analysis of these data revealed a significant effect for PD 3.

3.3. Biochemical analysis of infected mice brain

The PrP^{Sc} accumulation in brains of compound treated and untreated terminally sick mice was analyzed by Western blotting. Western blot analysis did not detect any differences in the glycosylation pattern or molecular weight of PrP^{Sc} between PD 1–4 treated and control mice (Fig. 3). In addition the quantification of the

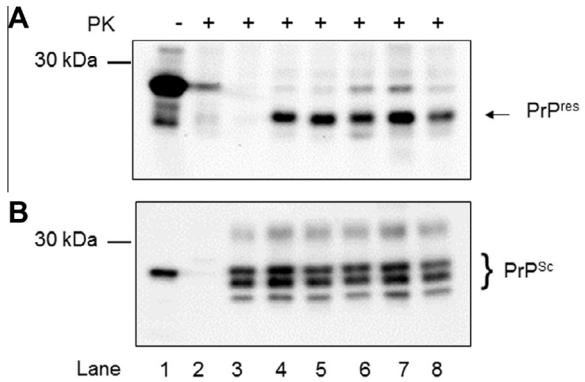


Fig. 1. Western blot analysis of inhibition of PrP^{res} formation and disaggregation of pre-existing PrP^{Sc} fibrils in a cell-free conversion assay. Piperazine derivatives do not interfere with cell-free PrP^{res} formation and do not dissolute existing PrP^{Sc}-aggregates: (A) controls were carried out as follows: PrP^{C} in a 1:10 dilution without PrP^{Sc} or PK (lane 1), PrP^{C} without PrP^{Sc}, incubated with PK (lane 2) and PrP^{C} incubated with PrP^{Sc}, but stopped immediately by freezing (t_0 control, lane 3). Lane 4 shows the PrP^{res} fragments after incubation for 72 h with Me7, in the presence of 100 μg/ml PD-1 (lane 5), 100 μM PD-2 (lane 6), 100 μM PD-3 (lane 7) and 100 μM PD-4 (lane 8). Detection of PrP^{C} and PrP^{res} fragments was carried out with mab 3F4. (B) The membrane in panel A was stripped and re-incubated with pab Ra10 for the detection of PrP^{Sc} seeds (mouse passaged scrapie strain Me7) in lanes 3–8. Western blot was analyzed by standard chemiluminescence method. +: incubation with proteinase K (PK).

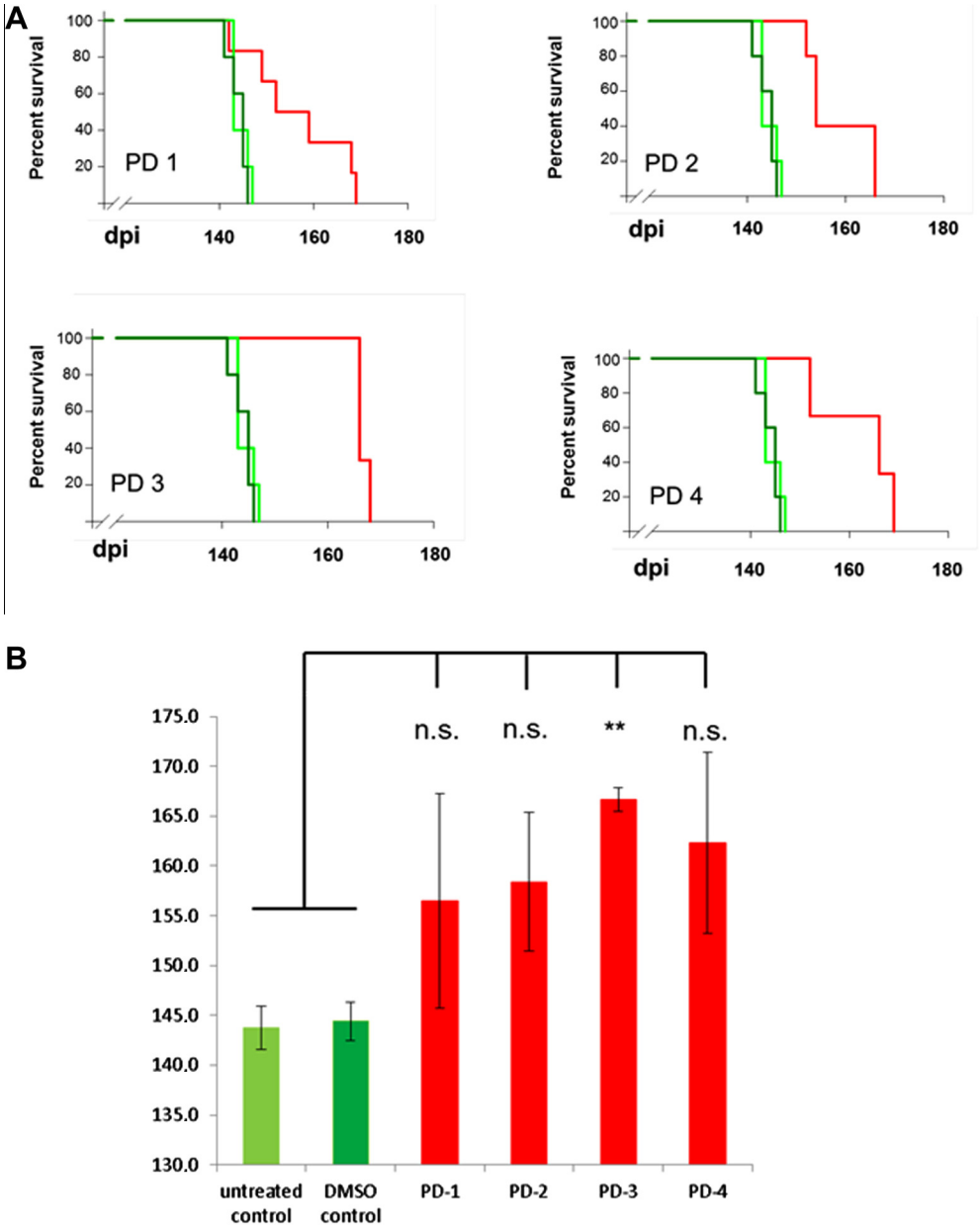


Fig. 2. Piperazine derivatives prolong the prion disease incubation time in scrapie infected mice after i.p. treatment. (A) Kaplan–Meier survival analysis of i.c. scrapie infected C57BL/6 mice was performed after i.p. treatment with PD 1–4. The treatment groups included untreated controls (lime green line), DMSO controls (olive green line) and PD 1–4 treated mice (red line). (B) Mean survival times ± the standard deviations as determined by the primary efficiency test. Comparison of PD 1–4 versus untreated control and DMSO control was carried out by using the log-rank test ($^{**}P < 0.01$, n.s. not significant).

Western blot results confirmed that the amount of PrP^{res} accumulation was similar in untreated and PD 1–4 treated mice (Fig. 3).

4. Discussion

The screening of 10,000 compounds from a chemical compound library (DIVERSet 2, ChemBridge) by using a cell-based assay identified six different inhibitor classes. Four of them (diphenylpyrazoles, benzimidazoles, quinolonesandindoles) have been already evaluated as antiprion compounds: diphenylpyrazole derivatives prolonged survival time in scrapie infected mice [6] and showed efficacy in *in vitro* and *in vivo* models for synucleinopathies like Parkinsons's Disease [7]. Benzimidazole derivatives inhibited PrP^{Sc} accumulation and prion infectivity in scrapie infected cells [20]. Attachment of scaffolded quinolone derivatives [21] as well as indole substructures [22]also decreased PrP^{Sc} levels in cell culture. Finally, benzothiophen derivatives like T-817MA showed beneficial effects in other neurodegenerative diseases, inhibited the neuronal damages and improved the motor and cognitive impairments in a mouse model for Huntington disease [23].

The most effective compound class *in vitro*, however, were piperazine derivatives. Three out of four substances (PD-1, PD-2 and PD-4) were analogues of aryl-piperazine and displayed IC_{50} values between 0, 1 and 0.2 μM and showed similar anti-prion activities like aryl-piperazine compounds previously reported [24].When they were injected i.p., a general prolongation of the incubation time of 13–18 days in scrapie infected mice was found. However, the prolongation was not significant when performing a Kaplan–Meier Survival analysis. In contrast, the fourth compound PD 3, a piperazine analogue having pyridine and indane substitu-

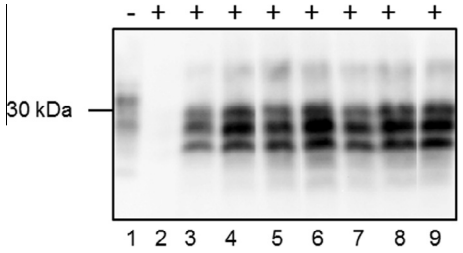


Fig. 3. PrP^{Sc} accumulation in the brains of selected mice challenged with RML. Control experiments were carried out using PrP^{C} without PK (lane 1), PrP^{C} digested with PK (lane 2) and PrP^{Sc} digested with PK (lane 3). Lanes 4 and 5 show PrP^{Sc} fragments of an untreated control mouse (lane 4) or of a dissolving solution mouse (lane 5). Lanes 6–9 show PrP^{Sc} fragments derived from intracerebrally inoculated mice, treated with PD 1 (lane 6) or with PD 2 (lane 7) or with PD 3 (lane 8) or with PD 4 (lane 9). Detection of PrP^{C}/PrP^{Sc} was carried out with mab SAF70 and mab RGS-His for the detection of the molecular mass markers.

ents (1-(2.3-dihydro-1H-inden-2-yl)-4-(2-pyridinyl) piperazine) displayed a IC_{50} value of 0, 4 μM but significantly prolonged the incubation time by 23 days. All components were administered i.p. in these assays which meant that they had to overcome the blood–brain barrier to reach their site of therapeutic action. Further lead structure design on the PD 1–4 may therefore improve their efficacy by boosting the bioavailability in the brain. Inbibitory effects observed here were not based on direct PrP interference nor PrP^{Sc} dissolution, since cell-free conversion of prion protein is not affected by these compounds. Moreover, the substances did not affect cellular trafficking and processing of PrP^{C} in N_2a cells and did not interfere with the protein degradation machinery, because proteasome activity was not affected.

In contrast, similar piperazine derivatives based on a 2–5 diketopiperazine scaffold interact directly with recombinant prion protein (recPrP), inhibited its aggregation and reduced PrP^{Sc} levels in scrapie infected mouse cells [25]. However the therapeutic evaluation of the compound class *in vivo* is still lacking.

Therefore, the exact molecular mechanism of the piperazine derivatives activity remains unresolved. It is likely that PD 1–4 have an indirect inhibitory mechanism in scrapie-infected cells.

According to this, a possible mode of action would be the interaction of piperazines to potential co-factors of the protein aggregation, as it was shown for Alzheimer's disease (AD): recently it was shown, that piperazine naphthamide derivatives block the formation of Aβ peptides by specifically inhibiting amyloid precursor protein cleavage enzyme (BACE-1) [26]. Other compounds, that potentially inhibit the decrease of acetylcholine in AD affected brains, such as donepezil, are 2-(2-(4-benzylpiperazin1-μl)ethyl) isoindoline derivatives, which effectively block acetylcholinesterase (AChE) *in vitro* [27].

In this context the piperazine derivatives PD 1–4 are suspected to target cofactors of prion misfolding or aggregation. This may explain the lack of inhibition in the cell-free conversion assay, which deals only with the interaction of purified prion protein and aggregates devoid of additional components. Potential targets are chaperones that modulate prion misfolding and aggregation as well as apoptosis. One of them – heat shock protein (hsp) 70 – is known to prevent improper folding and aggregation of PrP^{Sc} aggregates and to reduce its neurotoxicity in neurons [28].

Taken together, piperazine derivatives can be considered as promising lead structures for the treatment of prion diseases. Further studies are planned to determine their mode of action.

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