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Diphenylpyrazole-Derived Compounds Increase Survival Time of Mice after Prion Infection[▽]

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Transmissible spongiform encephalopathies (TSEs) represent a group of fatal neurodegenerative disorders that can be transmitted by natural infection or inoculation. TSEs include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jakob disease (CJD) in humans. The emergence of a variant form of CJD (vCJD), which has been associated with BSE, produced strong pressure to search for effective treatments with new drugs. Up to now, however, TSEs have proved incurable, although many efforts have been made both *in vitro* and *in vivo* to search for potent therapeutic and prophylactic compounds. For this purpose, we analyzed a compound library consisting of 10,000 compounds with a cell-based high-throughput screening assay dealing with scrapie-infected scrapie mouse brain and ScN$_2$A cells and identified a new class of inhibitors consisting of 3,5-diphenylpyrazole (DPP) derivatives. The most effective DPP derivative showed half-maximal inhibition of PrP^{Sc} formation at concentrations (IC$_{50}$) of 0.6 and 1.2 μM, respectively. This compound was subsequently subjected to a number of animal experiments using scrapie-infected wild-type C57BL/6 and transgenic Tga20 mice. The DPP derivative induced a significant increase of incubation time both in therapeutic and prophylactic experiments. The onset of the prion disease was delayed by 37 days after intraperitoneal and 42 days after oral application, respectively. In summary, we demonstrate a high *in vitro* efficiency of DPP derivatives against prion infections that was substantiated *in vivo* for one of these compounds. These results indicate that the novel class of DPP compounds should comprise excellent candidates for future therapeutic studies.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are incurable neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) in humans (62), chronic wasting disease in cervids (CWD), scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in bovines (15, 54). Human prion diseases include sporadic CJD, inherited forms such as Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia, and also acquired forms, which are caused by accidental inoculation (iatrogenic CJD) or by ingestion (kuru) of infectious material (14). During the last decade a variant form of CJD (vCJD) in young adults has been reported (2, 29). After transmission to mice (6) and primates (40), both neuropathological data and biochemical results for the pathological prion protein yielded compelling evidence for a link between BSE and vCJD.

Although the mechanism remains unclear, the central process in TSEs is the conversion of the physiological cellular prion protein (PrP^C) into a disease-associated isoform (PrP^{Sc}) (5, 47, 52). PrP^C represents a membrane-associated, detergent-soluble, and proteinase K (PK)-sensitive glycoprotein of unknown function (9, 10). During the conversion, PrP^C is transformed into a PK-resistant isoform, with a high content of β-sheet structures, which forms detergent-insoluble aggregates and deposits in the brain (31, 51).

Until now, TSEs have proved incurable; therefore, a main objective of this project was to search for potent therapeutic and prophylactic compounds. A large number of compounds were found to inhibit the PrP^{Sc} formation with high efficiency *in vitro*, such as tannic acid (34), curcumin (11, 36), or antimalaria drugs such as mefloquine (37). These compounds interact directly with PrP^{Sc} aggregates, but no *in vivo* activity could be proven. Other effective compounds were toxic (such as Congo red [7]), displayed only poor penetration of the blood-brain barrier (56), or were only transiently effective (such as RNA interference [17]). Potent inhibitors which act *in vitro* and *in vivo* are polyene antibiotics such as amphotericin B (42, 49) and its analogues (1), tetracyclines (20), cyclic tetrapyrole-like porphyrins (12, 50), and polyanions such as pentosan polysulfate (PPS) (8, 21). Other therapeutic approaches were carried out through active immunization using prion peptides (41) or by passive immunization using monoclonal antibodies (60).

Several clinical trials with CJD patients were conducted using PPS progression (48, 61), acyclovir (18, 45), the antiviral drug amantadin (57), quinacrine (27), or flupirtine (46), but without significant effects on the progress of the disease.

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In order to find novel anti-prion compound with *in vivo* activity, we analyzed a large compound library using a cell-based high-throughput screening assa and both scrapie-infected wild-type C57BL/6 and transgenic Tga20 mice. Diphenylpyrazole (DPP) derivatives were discovered as new class of inhibitors that delayed the onset of the prion disease significantly after intraperitoneal or oral application.

MATERIALS AND METHODS

Chemicals. A compound library (DIVERset 1) was purchased from Chem-Bridge Corp. (San Diego, CA), and compounds were diluted to 1 to 2 mM stock dilutions in dimethyl sulfoxide (DMSO). For the *in vivo* experiments, the compounds were either dissolved according to their solubility in 100 or 70% DMSO or were suspended in physiological saline (0.9% NaCl) just before use.

Testing for inhibition of PrP^{Sc} accumulation in a cell-based dot blot model. Approximately 20,000 mouse neuroblastoma (ScN$_2$a) or mouse brain (SMB) cells infected with the Rocky Mountain Laboratory scrapie strain (RML) were suspended in 100 µl of medium and seeded into a Costar 3599 flat-bottom 96-well plate (Corning, Inc., Corning, NY) prior to the addition of the test compound. The 1 to 2 mM solutions of the test compounds were diluted in DMSO prior to being added to the cell medium. A 1-µl portion of each solution was added to the cell medium. After incubation at 37°C for the ScN$_2$a cells and at 35°C for the SMB cells in a CO$_2$ incubator 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 compound 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), we transferred the cell lysate to an activated polyvinylidene difluoride membrane (Immobilon-P; Millipore) under vacuum pressure and fixed it to the membrane for 1 h at 37°C. The membrane was then incubated simultaneously in lysis puffer and treated with a 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 guanidinium thiocyanate, 0.1 M Tris-HCl [pH 8]) was added for 10 min at room temperature and, afterward, the membrane was washed five times with pure water. After the denaturation the membrane was blocked with 5% (wt/vol) nonfat milk containing 0.1% (vol/vol) Tween 20 (Sigma) in phosphate-buffered saline (PBST-milk) for 60 min. The polyclonal antibody (PAb) Ra10 (26, 28) was incubated in the blocking solution with the membrane for 60 min. After three rinses in PBST, the membrane was incubated in 0.2 µg of horseradish peroxidase-conjugated, anti-rabbit IgG (Promega)/ml in PBST-milk at room temperature for 1 h. After an additional rinse 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 ScN$_2$a and SMB cells, were analyzed.

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 µM to 2.0 µM and down to 0.2 µM) of each compound to the corresponding cell line.

Testing for PrP^C expression in uninfected N$_2$a cells. Approximately 20,000 N$_2$a cells were seeded into 96-well-plates. Further processing including compound application, cell lysis and PrP^{Sc} detection was carried out as described earlier.

MTT test. We performed an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] test (Sigma) as a marker for cell viability. Therefore, about 20,000 N$_2$a cells were added to each well of a 96-well plate. Test compounds were added to a final concentration of 20 µM for 3 days. After that, the MTT test was conducted according to the manufacturer's instructions. In this test, the tetrazolium salt MTT was converted into blue insoluble formazan dye crystals, which had to be dissolved by a suitable extraction mixture. The formazan absorption was quantified through spectrophotometric analysis (570-nm test wavelength; 630-nm reference wavelength).

Proteasome-Glo cell-based assay. Approximately 20,000 N$_2$a cells were added to each well of a 96-well plate. The cells were allowed to settle before the test compounds were added to a final concentration of 20 µM and incubated for 3 days. After that, a Proteasome-Glo assay was conducted according to the man-

ufacturer's instructions (Promega). In this assay, we measured the activity of the proteasome complex in cultured N$_2$a cells by cleavage of a luminogenic proteasome substrate, Suc-LLVY-aminoluciferin.

Cell-free conversion assay. This assay was carried out according to the protocol established by Eiden et al. (22) and Kupfer et al. (39). In brief, cell-free conversion was carried out by mixing bacterial recombinant murine 3F4-tagged prion protein 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 (33). 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 analyzed with regard to inhibition of PrP^{res} formation and dissolution of PrP^{Sc} aggregates.

PrP^{Sc} detection. Detection of PrP^{Sc} aggregates was examined by phosphotungstic acid (PTA) precipitation and Western blotting, which were carried out according to the protocols established by Wadsworth et al. (59) and Gretzschel et al. (25), with some modifications.

For all samples, 10% (wt/vol) brain homogenates were prepared in 0.42 mM sucrose solution containing 0.5% DOC and 0.5% Nonidet P-40 using a Ribolyser (Hybaid, Heidelberg, Germany). Large cellular debris was removed by centrifugation at 6,000 rpm for 5 min at room temperature. A 200-µl aliquot of the supernatant was adjusted with PK (Boehringer Mannheim) to a final concentration of 50 µg of PK/ml, followed by incubation at 55°C for 1 h. Digestion was stopped by the addition of 4 µl of Pefabloc (Roche, Mannheim, Germany), followed by heating to 95°C for 5 min. In the next step, PTA was added to the samples to give a final concentration of 0.3% (wt/vol). The samples were incubated at 37°C for 60 min with constant agitation before centrifugation at 13,300 rpm for 30 min at room temperature. After careful removal of the supernatant, the pellets were resuspended in various volumes of a loading buffer containing 1% (wt/vol) sodium dodecyl sulfate (SDS), 25 mM Tris-HCl (pH 7.4), 0.5% mercaptoethanol, and 0.001% bromophenol blue and then heated for 5 min at 95°C. The volume of loading buffer was adjusted in order to ensure comparable strengths in the Western blot signals. After a short centrifugation, samples were loaded on 16% Tris-polyacrylamide gels. SDS-PAGE and Western blotting was performed according to standard procedures (22). As a primary antibody, we used MAb SAF70 (Spibio) at a concentration of 0.03 µg/ml.

Immunohistochemistry. All tissues samples were fixed in 4% buffered formalin, treated for 1 h with 98% formic acid, rinsed for 40 min in tap water and embedded in paraffin. The paraffin-wax tissue sections were mounted on Superfrost Plus slides (Menzel-Gläser) and rehydrated. The subsequent pretreatment included incubation of the slides in 98% formic acid for 15 min, a 5-min rinse in tap water, and inhibition of the endogenous peroxidase activity with 3% H$_2$O$_2$ (Merck) in methanol for 30 min, followed by a 15-min digestion with PK (4 mg/ml; Boehringer Mannheim) at 37°C. The primary MAb ICSM 18 (D-Gen, Ltd.) was applied at a dilution of 1:250 in goat serum, followed by incubation at room temperature for 2 h. Negative-control sections were treated with goat serum alone. As a secondary antibody system, we used the EnVision reagent (Dako) containing a peroxidase-conjugated polymer backbone. The incubation time was 30 min at room temperature. The slides were finally developed in diaminobenzidine tetrahydrochloride (Fluka) and counterstained for 10 min with Mayer's hematoxylin. All sections were examined by light microscopy.

Propagation of scrapie strains. Wild-type C57BL/6 or transgenic Tga20 mice (kindly provided by C. Weissmann, Zürich, Switzerland) which overexpress wild-type mouse PrP and have shortened incubation periods for mouse prions (23) were inoculated either intracerebrally (i.c.) with 30 µl of a 1% homogenate of RML or intraperitoneally (i.p.) with 50 µl of a 1% RML homogenate. The health status of the mice was inspected daily, and their body weights were recorded weekly. After the onset of TSE-associated clinical symptoms (abnormal tail tonus, hind limb paralysis, and weight loss), the animals were euthanized. The incubation time was then calculated as the time between inoculation and death. The brains were removed; 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 intercurrent diseases were excluded from the data. Table 2 gives an overview about the animal experiments.

Toxicity study. The toxicity of DMSO solved 2-[5-(3-fluorophenyl)-1*H*-pyrazol-3-yl]-5-methylphenol (DPP-1; 5 mg/kg/day) was analyzed by application to uninfected C57BL/6 mice.

Therefore, 100 µg of DPP-1 (5 mg/kg/day) was administered i.p. to five wild-type mice daily for a period of 21 days. In addition, five mice were used as dissolving-solution controls, and six mice were left untreated. Abnormalities that might be due to the compound administered were examined by pathological and

TABLE 1. Chemical structures off DPP derivatives and *in vitro* analyses

Compound	Dot blot (SMB and ScN$_2$a)[a]	IC$_{50}$ (μM)[b]		Analyses[c]			
		SMB	ScN$_2$a	MTT test	Inhibition of PrP-expression in N$_2$a cells	Inhibition in cell-free assay	Influence of proteasome activity
(structure: OH, N—NH pyrazole, F, H$_3$C)	(blots)	0.6	1.2	NT	NInh	NE	NInf
(structure: OH, N—NH pyrazole, F)	(blots)	20	1.95	NT	NInh	NE	NInf
(structure: OH, N—NH pyrazole, Br)	(blots)	25	24	NT	NInh	NE	NInf

[a] Left blot, SMB; right blot, ScN$_2$a.
[b] IC$_{50}$, half-maximal inhibitory concentration.
[c] NT, nontoxic; NInh, no inhibition; NE, no effect; NInf, no influence.

histopathological analysis of the most relevant tissues (i.e., the liver, kidneys, brain, lungs, heart, muscles, gut, genitalia, spleen, and pancreas).

Intraperitoneal injection. The daily treatment with 100 μg (5 mg/kg/day) of the DPP-1 compound dissolved in 100% DMSO started 90 days after the i.c. inoculation of C57BL/6 mice with RML and was administered i.p. over a period of 20 days (Primary efficiency test). As a control, inoculated mice were injected with pure DMSO and were used as dissolving solution controls (DMSO-control). For treatment with a benzothiazol derivative (BTD), [2-methoxy-4-({[4-(6-methyl-1,3-benzothiazol-2-yl)phenyl]imino}methyl)phenol, the BTD was dissolved in 100% DMSO and administered i.p. Daily treatment with 100 μg of BTD started 14 days after i.p. inoculation of C57BL/6 mice with RML over 8 weeks.

Osmotic pump treatment. Application of DPP compound was first carried out with osmotic pumps (Alzet, Cupertino, CA), which allowed a continuous administration of the compound over a period of 8 weeks. The DPP compound was dissolved in 70% DMSO (maximum capacity for the osmotic pump membrane) The polyethylene catheter of the pump was inserted into the peritoneum, and the compound was administered over 4 weeks at a flow rate of 0.25 μl/h, which corresponded to 100 μg per mouse per day. The pump worked stably for 40 h after implantation and continuously for 4 weeks. After 4 weeks the pump was replaced by another pump, and the procedure was continued for another 4 weeks. This drug delivery schedule allowed a continuous and undisturbed administration of the test agents without the need for perpetual injections. As a control, 70% DMSO was administered via osmotic pumps (vehicle control).

Oral treatment. In terms of the oral application approach, we resuspended DPP-1 and BTD in 0.9% NaCl to a concentration of ~5 mg/ml, which led to a daily dose of 500 μg per mouse. The administration was carried out with feeding needles (Scanbur, BK, Sollentuna, Sweden).

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.

RESULTS

***In vitro* testing.** To discover efficient anti-prion compounds in a library of 10,000 chemical substances (DIVERset 1; ChemBridge), we established a high-throughput screening assay for PrP^{Sc} inhibitors using ScN$_2$a cells and SMB cells in a 96-well format. Using these *in vitro* systems and a computerized representation of the data, we found a new class of inhibitors comprising 3,5-diphenylpyrazole (DPP) derivatives by structure-activity and cluster analysis (30).

Among these, 2-[5-(3-fluorophenyl)-1*H*-pyrazol-3-yl]-5-methyl-phenol, (here designated DPP-1), 2-[5-(3-fluorophenyl)-1*H*-pyrazol-3-yl]phenol (DPP-2), and 2-[5-(3-bromophenyl)-1*H*-pyrazol-3-yl]phenol (DPP-3) were the most effective PrP^{res} formation inhibitors. Structures, dot blot results and IC$_{50}$s of all relevant compounds are summarized in Table 1.

DPP-1 exhibited IC$_{50}$s of 0.6 μM in SMB cells and 1.2 μM in ScN$_2$a cells, whereas the IC$_{50}$s of DPP-2 were 20 μM (SMB cells) and 19.5 μM (ScN$_2$a cells), respectively, and the IC$_{50}$s of
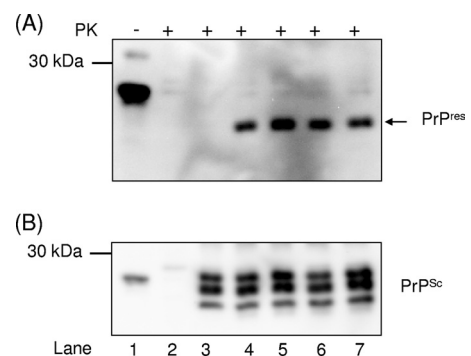

FIG. 1. DPP derivatives do not interfere with cell-free PrP^{res} formation and do not dissolve existing PrP^{Sc} aggregates. (A) Controls were performed as follows: PrP^C in a 1:10 dilution without PrP^{Sc} or PK (lane 1), PrP^C without PrP^{Sc} incubated with PK (lane 2), and PrP^C incubated with PrP^{Sc} but stopped immediately by freezing (t_0 control, lane 3). Lane 4 shows the PrP^{res} fragments after incubation for 72 h with scrapie strain Me7 in the presence of 100 μM DPP-1 (lane 5), 100 μM DPP-2 (lane 6), or 100 μM DPP-3 (lane 7). Detection of PrP^C and PrP^{res} fragments was carried out with MAb 3F4. (B) The membrane in panel A was stripped and reincubated with PAb Ra10 for the detection of PrP^{Sc} seeds in lanes 3 to 7.

TABLE 2. Animal experiments

Approach	Treatment	Mice	Inoculation
Primary efficiency test	i.p.	C57BL/6	i.c.
Therapeutic approach	i.p.	Tga 20	i.p.
	Oral	Tga 20	i.p.
Prophylactic approach	Oral	C57BL/6	i.p.

DPP-3 were 25 μM (SMB cells) and 24 μM (ScN$_2$a cells). Moreover, all of the compounds lacked cytotoxic effects, as shown by the MTT test.

Further analysis of any potential molecular inhibitory mechanism was performed using a cell-free conversion assay. All three compounds had no influence on the PrP^{res} formation (Fig. 1A) or on the dissolution of PrP^{Sc} aggregates (Fig. 1B). We were also unable to detect any influence of these compounds on the proteasome activity, as well as on the cellular trafficking and processing of PrP^C in N$_2$a cells. Based on its high inhibitory effects for scrapie-infected cells, DPP-1 was selected to be used in *in vivo* studies.

In vivo testing. The anti-prion efficacy of DPP-1 was determined by mouse bioassays using C57BL/6 wild-type and transgenic Tga20 mice. The experimental approach included a toxicity study, as well as three treatment regimes (primary efficiency, prophylactic, and therapeutic) for the mice (Table 2).

Toxicity study. Following a substantial DPP dose (i.e., 5 mg/day i.p. for a period of 21 days), no pathoanatomical and histopathological alterations were found. The only affections that were observed were a fibrous peritonitis in some of the DPP-1 treated mice, as well as in the DMSO control mice, which was most probably caused by the application.

Primary efficiency test. The results of this experiment are summarized in Fig. 2A and B. Untreated control mice had a mean incubation time of 160 ± 4.1 days, which was similar to that of the DMSO control mice (160 ± 6.5 days). DPP-1 significantly prolonged the mean incubation time for 14 days (from 160 ± 4.1 days to 174 ± 3.8 days). Based on these findings, the therapeutic and prophylactic effects of DPP-1 were further evaluated in animal experiments. Moreover, we included a BTD for which we have recently found an anti-prion effect *in vitro* and *in vivo* (24).

Therapeutic approach. In a first set of experiments, a therapeutic approach was applied using transgenic Tga20 mice that had been i.p. challenged with the RML strain. DPP-1 was administered by continuous subcutaneous infusion using osmotic pumps. Under these conditions DPP-1 prolonged the mean incubation time of scrapie in the mice by 37 days compared to that in untreated control mice (from 113 ± 6.4 days to 149 ± 3 days). The mean value for the vehicle control (70% DMSO) was 112 ± 6.6 days (Fig. 2C and D).

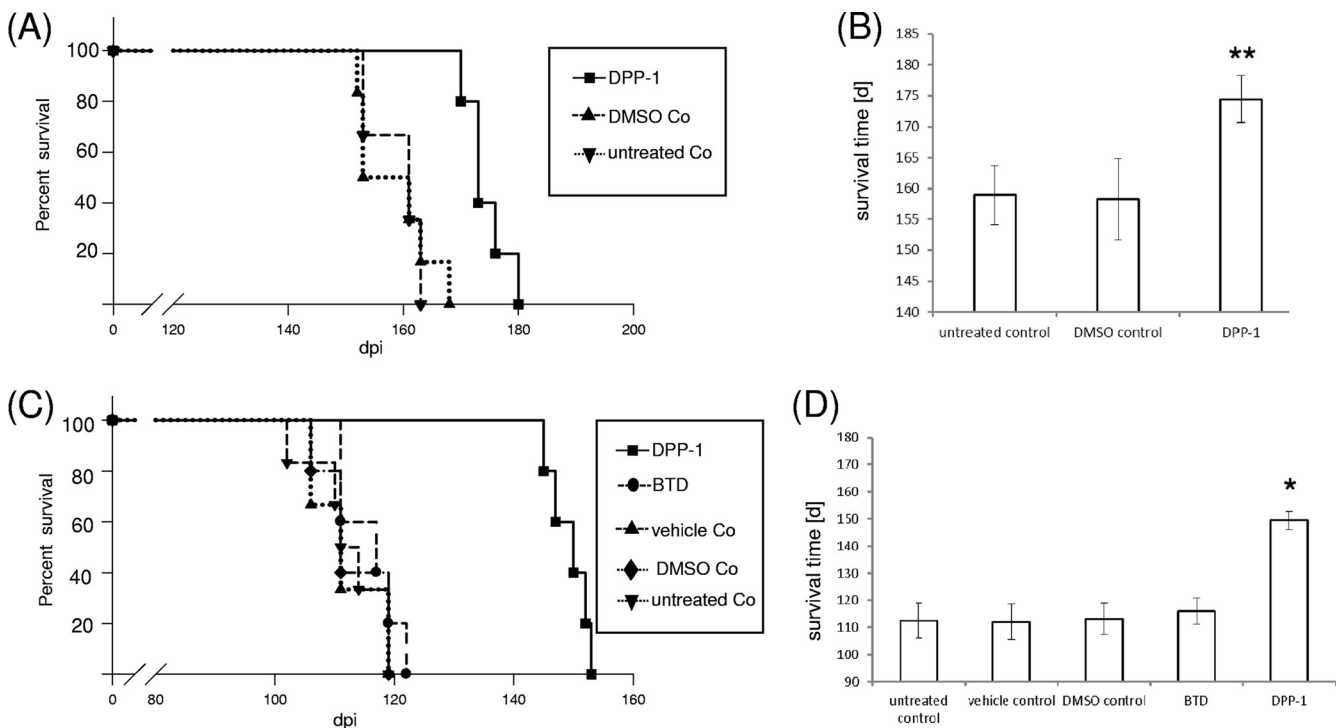Portions (100 μg) of BTD (per mouse and day) were given

FIG. 2. DPP-1 prolongs the incubation time in scrapie-infected mice after i.p. treatment. (A) Primary efficiency test. Kaplan-Meier survival analysis of i.c. scrapie-infected C57BL/6 mice was performed after i.p. treatment with DPP-1. The treatment groups included untreated controls (▼), DMSO controls (▲), and DPP-1-treated mice (■). (B) Mean survival times ± the standard deviations as determined by the primary efficiency test. Comparison of DPP-1 versus untreated controls and DMSO controls was carried out by using the log-rank test (**, $P < 0.01$). (C) Intraperitoneal therapy. Kaplan-Meier survival analysis of i.p. scrapie-infected Tga20 mice was performed after i.p. treatment with BTD or DPP-1. The treatment groups included untreated controls (▼), vehicle controls (▲), DMSO controls (◆), benzothiazol (BTD)-treated mice (●), and DPP-1-treated mice (■). (D) Mean survival times in i.p. therapy ± the standard deviation. Comparison of DPP-1-treated versus untreated controls, DMSO controls, vehicle control, and BTD-treated mice was carried out by using the log-rank test (*, $P < 0.05$).
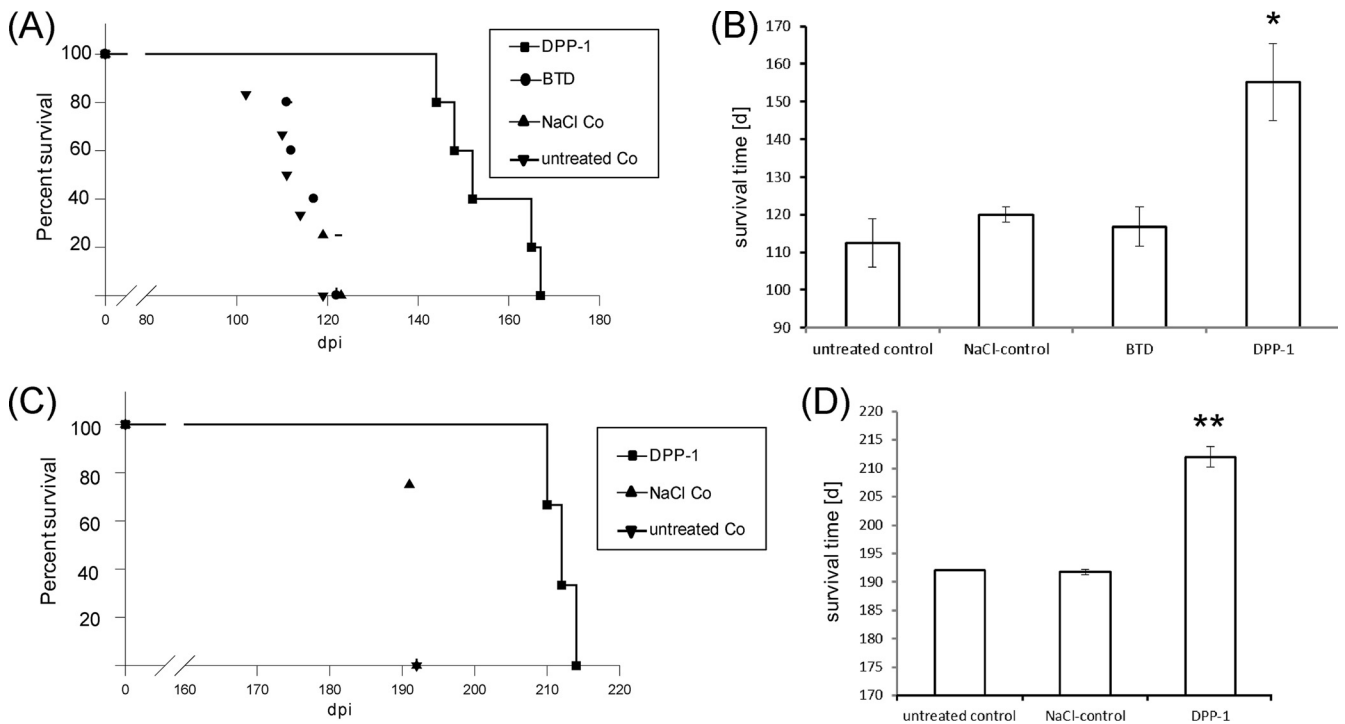
FIG. 3. DPP-1 prolongs incubation time in scrapie-infected mice after oral treatment. (A) Oral treatment. Kaplan-Meier survival analysis of i.p. scrapie-infected Tga20 mice was performed after oral treatment with DPP-1 or benzothiazol (BTD). The treatment groups included untreated controls (▼), NaCl controls (▲), benzothiazol (BTD)-treated mice (●), and DPP-1-treated mice (■). (B) Mean survival times in primary efficiency test ± the standard deviation. Comparison of DPP-1 versus untreated controls, NaCl controls, and BTD-treated mice was carried out by using the log-rank test (∗, $P < 0.05$). (C) Oral prophylaxis. Kaplan-Meier survival analysis of i.p. scrapie-infected C57BL/6 mice was performed after oral treatment with DPP-1. The treatment groups included untreated controls (▼), NaCl controls (▲) and DPP-1-treated mice (■). (D) Mean survival times in oral prophylaxis ± the standard deviation. Comparison of DPP-1-treated versus untreated controls and NaCl controls was carried out by using the log-rank test (∗∗, $P < 0.01$).

daily by i.p. injection since the BTD is soluble only in 100% DMSO, which is not tolerated by the osmotic pumps. Application of the DMSO control without BTD had no effect on the incubation time (113 ± 5.6 days) compared to the untreated control (113 ± 6.4 days) and also the treatment with benzothiazol caused only a minor prolongation of the incubation time (116 ± 5 days) (Fig. 2C and D).

In a second set of experiments DPP-1 and benzothiazol were administered daily to Tga20 mice by using oral feeding needles over a period of 8 weeks, starting 14 days after i.p. inoculation. The animals treated with benzothiazol showed a mean incubation time of 117 ± 5.0 days, and the NaCl control group a mean value of 120 ± 2 days. In contrast, treatment with DPP-1 significantly increased the incubation time by 42 days (155 × 10) compared to the untreated control (113 ± 6.4 days) (Fig. 3A and B).

In conclusion, the *in vitro* inhibitory activity of benzothiazol was not confirmed *in vivo*, whereas a DPP-1 treatment increased in the scrapie incubation time by ∼37 days (osmotic pumps) or 42 days (oral treatment), respectively. Therefore, DPP-1 should be considered as promising drug candidate for the prophylactic treatment of prion diseases.

Prophylactic approach. An oral DPP-1 application started 2 weeks before the i.p. inoculation (RML) of wild-type C57BL/6 mice and continued for 6 weeks (overall time of treatment, 8 weeks). DPP-1 was again solubilized in 0.9% NaCl (5 mg/ml),

which yielded an intake of 500 μg of DPP-1 (25 mg/kg/day) per mouse effectively.

In this prophylactic approach, DPP-1 also increased the survival time significantly by 20 days compared to the controls (212 ± 1.8 days versus 192 ± 0.5 days) (Fig. 3C and D).

Biochemical and immunohistological analysis. The PrP^{Sc} accumulation in brains of compound-treated and untreated terminally sick mice was analyzed by Western blotting and by immunohistochemistry. Western blotting did not reveal any differences in the glycosylation pattern or molecular weight of PrP^{Sc} between DPP-1-treated and control mice (Fig. 4A and B). The same applied for benzothiazol (data not shown). The brain tissues of treated and untreated mice at the terminal stage of disease revealed typical disease specific PrP^{Sc} accumulation pattern (Fig. 4C and D).

DISCUSSION

The screening of 10,000 compounds from a chemical compound library by using cell-based assays identified DPPs as potent inhibitors of the PrP^{Sc} formation *in vitro*. The IC$_{50}$s of the most efficient inhibitor (DPP-1) varied between 0.6 μM in SMB cells and 1.2 μM in ScN$_2$a cells. The protective effect of DPPs was further analyzed in a cell-free conversion assay, but no effect was identified, which indicated an indirect inhibitory mechanism in scrapie-infected cells independent from the pro-
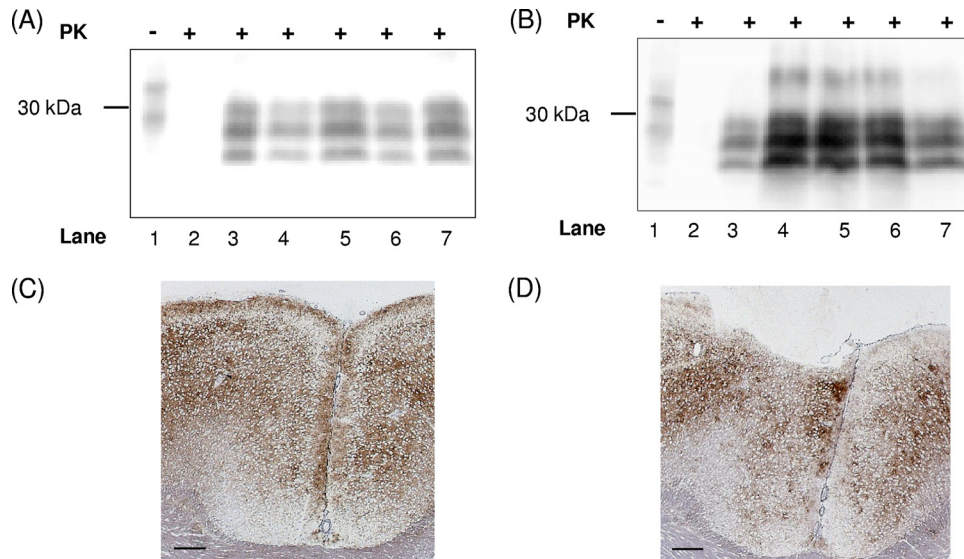
FIG. 4. PrP^{Sc} accumulation in the brains of selected mice challenged with RML. Immunoblot analysis of RML-infected mouse brain homogenate. (A) Control experiments were carried out using PrP^C without PK (lane 1), PrP^C digested with PK (lane 2), or PrP^{Sc} digested with PK (lane 3). Lanes 4 and 5 show PrP^{Sc} fragments of mice treated with osmotic pumps infused with DPP-1 (lane 4) or DMSO control (lane 5). Lanes 6 and 7 display PK-digested PrP^{Sc} fragments from orally treated mice (postinoculation) either with (lane 6) or without (lane 7) DPP-1. (B) Controls were established similar to those in Fig. 4A: PrP^C without K (lane 1), PrP^C digested with PK (lane 2), or PrP^{Sc} digested with PK (lane 3). Lanes 4 and 5 show PrP^{Sc} fragments derived from i.c.-inoculated mice treated with DPP-1 (lane 4) or not treated (lane 5). Lanes 6 and 7 display PK-digested PrP^{Sc} fragments of i.p.-infected mice after oral prophylactic administration of DPP-1 (lane 6) compared to the NaCl controls (lane 7). Detection of PrP^C or PrP^{Sc} was carried out with MAb SAF70 and MAb RGS-His for the detection of molecular mass markers. PrP^{Sc} immunohistochemistry of brain tissue was performed. (C and D) Cortices of DPP-1-treated (C) and untreated (D) i.p.-infected C57BL/6 mice (oral prophylaxis) at the terminal stage of disease. MAb ICSM 18, 1:250; bar, 200 μm.

tein degradation machinery, because the proteasome activity *in vitro* was not affected. MTT tests as a marker for cell viability showed no *in vitro* toxicity of DPPs. Moreover, we examined also the DPP toxicity by mouse treatment *in vivo* and found no damaging effect. The efficacy of the most potent DPP derivative, DPP-1, was further analyzed in scrapie infected mice and a significant delay of clinical onset was observed. DPPs are most probably able to cross the blood-brain barrier, because the scrapie infection was performed i.c., whereas the compounds were administered i.p.

DPP-1 and another already published anti-prion compound, benzothiazol, were subsequently tested for their therapeutic activity. Scrapie-inoculated Tga20 mice (i.p.) received these compounds i.p. (via osmotic pumps or by i.p. injection) to reveal effects on the spread of the infection from the periphery to the central nervous system. In these experiments only DPP-1 extended the incubation time significantly (i.e., by 36 days).

Benzothiazol derivatives have been shown to effectively bind adenosine receptors and antagonize the neuromodulator adenosine (Hoffmann-La Roche AG, document DE602004004776T2 22.11.2007, EP publication no. 0001628662). Adenosine receptors are involved in the control of neuronal damage and therefore display a specific target for the treatment of neurodegenerative disorders (16). Benzothiazol may therefore only induce general beneficial effects with regard to neuroprotection and neuroinflammation.

Further therapeutic and prophylactic DPP-1 treatments were carried out orally, and significant prolongations of the incubation period were revealed. In contrast, Benzothiazol did not show any effect.

The general molecular mechanisms of prion inhibition by DPPs are unknown to date. DPPs can act as inhibitors of heat shock proteins (Hsps), especially Hsp90 (H. Eggenweiler, M. Wolf, and Merck Patent GmbH, 1,5-Diphenylpyrazoles, WIPO patent application WO/2006/018082, 2005). Hsps belong to the group of molecular chaperones that regulate protein folding and therefore play an essential role during neurodegeneration (55). Normally, chaperones refold damaged proteins or target them to degradation. Under specific conditions, however, they transfer damaged proteins to larger aggregates. This was previously demonstrated for the chaperones GroEL and Hsp104, which stimulated the PrP conversion (19).

On the other hand, inhibition of Hsp90 induces the upregulation of Hsp70 and other Hsps that inhibit the formation of toxic protein aggregates (53). Recently, the inhibition of Hsp90 by novobiocin analogues has been reported to suppress amyloid beta-induced neurodegeneration (3). A similar effect has been demonstrated with the Hsp90 inhibitor geldanamycin, which decreases α-synuclein aggregation in cell culture (44). The interference to cofactors 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.

Another potential mode of DPP action is the role as antioxidant during the disease. The antioxidative potential of DPP derivatives with regard to inhibition of monoamine oxidase has been demonstrated in several studies (13, 43). During neurodegenerative diseases in mitochondria, an oxidation of numerous proteins by monoamine oxidase occurs. This event is followed by the generation of reactive oxygen species, which

ultimately leads to neuronal cell death. Thus, the beneficial effects of DPP may therefore result in a significantly prolonged incubation time in scrapie-infected mice, although the PrP aggregation continues to proceed.

Only a few other compounds have been reported that showed therapeutic effects against TSEs *in vivo*. PPS has been reported to decrease the PrP^{Sc} levels in prion-infected cells (4) and was further analyzed in scrapie-infected mice. Doh-ura et al. (21) showed that direct continuous PPS infusion over 4 weeks with the help of osmotic pumps at a dose of 230 μg/kg/day into the cerebral ventricle significantly prolonged survival time of intracerebrally scrapie infected mice. In addition, PPS was administered to CJD patients, but without observing therapeutic effects (61). Other compounds that have been shown to delay the onset of scrapie after i.c. inoculation were Congo red (32) and amphotericin B (49). In our study we could demonstrate that DPP-1 delayed the onset of disease by a daily i.p. injection (5 mg/kg/day) for 21 days starting 90 days after the i.c. scrapie challenge. In addition, we could monitor an anti-scrapie activity of DPP-1 in i.p.-infected mice when we used a continuous i.p. infusion by starting the therapy at an early stage of the infection (14 days postinoculation) at a dose of 5 mg/kg per day and mouse over 8 weeks. Recently, porphyrin derivates have been described to significantly increase the incubation time when administered i.p. 2 weeks prior to i.p. infection. However, these substances showed only little activity when administered to i.c.-inoculated mice (38). It must therefore be assumed that these compounds are unable to cross the blood-brain barrier.

Several attempts for an oral scrapie therapy had been carried out with previously analyzed *in vitro* inhibitors such as curcumin (11) and tannic acid, as well as phenolic tea extracts, even at doses of 1,500 mg/kg per day in drinking water (35). However, none of these substances displayed a convincing *in vivo* effect. Only the oral treatment with pravastin resulted in a mild prolongation of survival time (58). Pravastin, a hydrophilic member of the statins, is known to interfere with cholesterol biosynthesis pathway and harbors general anti-inflammatory and antioxidant effects.

Taken together, we report here on a new class of *in vivo* active anti-prion compounds, the 3,5-diphenylpyrazoles (DPPs are covered by patent application PCT/EP2009/004144), which work following i.p. and oral application using both therapeutic and prophylactic experimental setups. Based on our results, DPP-1 in particular should be considered as a novel promising candidate for intervention in prion diseases.

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