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Cellulose ether treatment *in vivo* generates chronic wasting disease prions with reduced protease resistance and delayed disease progression

Samia Hannaoui*, Maria Immaculata Arifin*, Sheng Chun Chang*, Jie Yu*, Preetha Gopalakrishnan*, Katsumi Doh-ura†, Hermann M. Schatzl* and Sabine Gilch* [iD]

*Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, Hotchkiss Brain Institute, University of Calgary, Calgary, Canada

†Department of Neurochemistry, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

Abstract

Chronic wasting disease (CWD) is a prion disease of free-ranging and farmed cervids that is highly contagious because of extensive prion shedding and prion persistence in the environment. Previously, cellulose ether compounds (CEs) have been shown to significantly extend the survival of mice inoculated with mouse-adapted prion strains. In this study, we used CEs, TC-5RW, and 60SH-50, *in vitro* and *in vivo* to assess their efficacy to interfere with CWD prion propagation. *In vitro*, CEs inhibited CWD prion amplification in a dose-dependent manner. Transgenic mice over-expressing elk PrP^C (tgElk) were injected subcutaneously with a single dose of either of the CEs, followed by intracerebral inoculation with different CWD isolates from white tailed deer, mule deer, or elk. All treated groups showed a prolonged survival of up to more than 30 % when compared to the control group regardless of the CWD isolate used for infection. The extended survival in the treated groups correlated with reduced proteinase K resistance of prions. Remarkably, passage of brain homogenates from treated or untreated animals in tgElk mice resulted in a prolonged life span of mice inoculated with homogenates from CE-treated mice (of + 17%) even in the absence of further treatment. Besides the delayed disease onset upon passage in TgElk mice, the reduced proteinase K resistance was maintained but less pronounced. Therefore, these compounds can be very useful in limiting the spread of CWD in captive and wild-ranging cervids.

Keywords: cellulose ether, chronic wasting disease, prions, prophylactic, PrP^{Sc} conformation, therapeutic.

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Prion diseases or transmissible spongiform encephalopathies are fatal progressive neurodegenerative disorders occurring in both humans and animals, caused by the accumulation of PrP^{Sc}. In humans, prion disease includes three types, sporadic Creutzfeldt–Jakob disease (CJD) which is the most common form, genetic disease, for example, familial CJD, and a type acquired through infection, for example, iatrogenic CJD or variant CJD (Wadsworth and Collinge 2007). Animal prion diseases include scrapie in sheep and goat, bovine spongiform encephalopathy in cattle, which ultimately caused vCJD upon zoonotic transmission to humans (Bruce *et al.* 1997), camel prion disease in dromedary camel (Babelhadj *et al.* 2018), and chronic wasting disease (CWD) in cervids (Watts *et al.* 2006).

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The "protein only" hypothesis states that prions are mainly composed of PrP^{Sc} (Prusiner 1982), an abnormally folded isoform of the cellular prion protein, PrP^C. The conversion of PrP^C, which has a predominantly α-helical structure, into a β-sheet enriched structure results in the PrP^{Sc} conformation (Wille *et al.* 2002; Groveman *et al.* 2014; Vazquez-Fernandez *et al.* 2016). The newly generated PrP^{Sc} acts as a seed, binds and converts other PrP^C molecules into new PrP^{Sc} molecules that are incorporated into a growing polymer (Caughey 2003; Soto *et al.* 2006). The latter breaks into smaller and more infectious nuclei, thus perpetuating the conversion cycle in an exponential and autocatalytic manner. PrP^{Sc} molecules have a high propensity for aggregation and are partially resistant to digestion with proteases such as proteinase K (PK) (McKinley *et al.* 1983; Prusiner 1998).

CWD affects free-ranging and farmed cervid species, including elk (*Cervus canadensis*), mule deer (MD; *Odocoileus hemionus*), white-tailed deer (WTD; *Odocoileus virginianus*), moose (*Alces alces*), and reindeer (*Rangifer tarandus tarandus*) (Williams and Young 1980; Gilch *et al.* 2011; Benestad *et al.* 2016). In CWD, PrP^{Sc} can be found in many extraneural tissues, body fluids, and excreta (Mathiason *et al.* 2006; Pulford *et al.* 2012; John *et al.* 2013). The shedding of prions into the environment makes the soil a reservoir of infectious prions for decades (Johnson *et al.* 2006; Smith *et al.* 2011; Saunders *et al.* 2012; Kuznetsova *et al.* 2014). CWD is considered highly contagious, with efficient horizontal transmission favored by cervid interactions and environmental persistence and uptake of infectious CWD prions (Johnson *et al.* 2007; Pritzkow *et al.* 2015). CWD is, with scrapie, one of the only two transmissible encephalopathies that is environmentally transmitted. Therefore, the impressive expansion of CWD in North America will most likely continue to grow. The geographic range of CWD expanded to 26 US states and 3 Canadian provinces in North America, as well as Norway, Finland, and Sweden in Northern Europe (CDC C. f. D. C. a. P. (2019)). Despite considerable efforts to develop an effective vaccine to contain CWD expansion (Pilon *et al.* 2013; Goni *et al.* 2015; Taschuk *et al.* 2017; Abdelaziz *et al.* 2017; Abdelaziz *et al.* 2018), the eradication of CWD seems not to be realistic to date. In addition, extended incubation periods, subtle early clinical signs, a persisting infectious agent in the environment, and incomplete understanding of transmission all constrain options for controlling CWD. CWD in farmed animals causes economic loss, increases the burden of environmental contamination and may be a reservoir for infecting free-ranging cervids. Altogether, these findings raise the necessity to develop management strategies to reduce transmission of CWD, both in farmed and free-ranging animals.

Recently, Teruya *et al.* reported remarkable anti-prion effects *in vivo* for cellulose ethers [CEs; (Teruya *et al.* 2016)]. CEs are hydrophilic macromolecules, which can be distributed throughout the whole body within days after a single subcutaneous injection. Teruya *et al.* described the crucial role of macrophages in the efficacies of CEs through phagocytosis, which facilitates the decomposition or excretion of CEs (Teruya *et al.* 2016). CE treatment significantly increased the survival of transgenic mice and hamsters, respectively, infected intracerebrally (i.c.) or intraperitoneally (i.p.) with 263K prions, even when only a single dose of CE was applied subcutaneously (s.c.) 1 year before prion infection. A single dose of s.c. applied CE was significantly effective in C57BL/6 mice infected i.c. with RML or Fukuoka-1 prions. However, the prolongation of incubation time was less pronounced than for 263K prions, leading to the question of the influence of prion strain and/or mouse genetic background on treatment outcomes. Mechanistically, CEs most likely inhibit prion formation (Teruya *et al.* 2016). Similarly, inhibition of α-synuclein amyloid formation (Breydo *et al.* 2014) *in vitro* was demonstrated, suggesting that CEs have a general inhibitory effect on amyloid formation.

Compounds such as CE that can be applied as single doses when cervids are handled could be used as a prophylactic treatment against CWD. In this study, we report the prophylactic anti-prion effects of CEs on CWD propagation *in vitro* and *in vivo*. *In vitro*, regardless of CWD isolates used as seeds, CEs showed an inhibitory effect on prion conversion in a cell-free conversion assay (real-time quaking-induced conversion, RT-QuIC). *In vivo*, a single s.c. injection of CEs had a protective effect against CWD infection, irrespective of the CWD isolate used for i.c. inoculation of transgenic mice over-expressing elk PrP^C [TgElk; (LaFauci *et al.* 2006)], whether CEs were administered the same day or 1 month prior to prion inoculation (group A and group B respectively).

In addition, we compared the biochemical properties of PrP^{Sc} between CE-treated and untreated mice. PK resistance of PrP^{Sc} from CE treated groups was significantly reduced, whereas upon guanidine hydrochloride denaturation PrP^{Sc} of all groups of mice followed the same denaturation profile. Interestingly, inoculation of TgElk mice with brain homogenates from CE-treated animals (group B) resulted in an extended survival, without further treatment, as compared to mice inoculated with brain homogenate from control mice. Delay of disease onset upon passage in TgElk mice was still associated to some extent to a reduced PK resistance of PrP^{Sc}. In addition, RT-QuIC data using brain homogenate from treated and non-treated mice as seeds indicated that no drug resistance developed, as the inhibitory effect of CEs in RT-QuIC reactions was similar for seeds from treated and non-treated mice, respectively.

In summary, these findings demonstrate that CEs interfere with prion propagation and extend the survival of mice inoculated with CWD isolates of different species by modifying some of PrP^{Sc} conformers' properties. Thus, CE

compounds are promising candidates for CWD prophylaxis. Their effect on prion infectivity may help to overcome CWD spreading and its consequences.

Materials and methods

This study was not pre-registered and no randomization was performed to allocate subjects in the study. All animal experiments were blinded to the experimenter during the animal experimentation, while there was no blinding for statistical analysis. There were no sample calculations or power analysis. Materials will be shared upon reasonable request.

Ethics statement

All animal use in the experiments strictly followed the guidelines of the Canadian Council for Animal Care. All experiments were performed in compliance with the University of Calgary Animal Care Committee under protocol numbers AC14-0122 (inoculation and treatment) and AC18-0030 (inoculation and treatment). The University of Calgary Animal Care Committee has approved the study prior to its starting.

Cellulose ethers

CE compounds TC-5RW and 60SH-50 were dissolved at a concentration of 5% in water. Compound properties and details are as described previously (Teruya *et al.* 2016).

CWD isolates

All isolates used in this study were prepared as 10% (w/v) brain homogenates in phosphate-buffered saline (PBS) pH 7.4 (cat #10010-023; LifeTechnologies, Gibco, CA, USA) using the MP Biomedicals fast prep-24 homogenizer (cat #116004500; Fisher, CA, USA). Aliquots were stored at −80°C. We used three different CWD isolates from CWD-infected elk, MD, and WTD. The CWD-elk isolate containing strain CWD2 was a pool of 3 CWD-elk (132MM) animals experimentally challenged by oral inoculation (Angers *et al.* 2010), kindly provided by Dr Stefanie Czub, Canadian Food Inspection Agency (Basu *et al.* 2012); the MD and WTD-116AG (Hannaoui *et al.* 2017a) isolates were from wild animals at the terminal stage of disease generously provided by Dr Trent Bollinger (University of Saskatchewan, Saskatoon, Saskatchewan, Canada).

Animal study

TgElk mice, a transgenic mouse line over-expressing elk PrP^C, was used to propagate different CWD isolates and was kindly provided by Dr Debbie McKenzie, University of Alberta (LaFauci *et al.* 2006). Prior to any treatments or inoculations isoflurane was used as anesthetic at a concentration of 5% (flow rate of 0.8 L/min) for induction, and then lowered to 0.5–1% for maintenance of general anesthesia. Because it is a safe gas to use for both animals and users, isoflurane is the preferred and commonly gas anesthetic agent in many animal facilities including ours (Taylor and Mook 2009). Six to eight weeks old female TgElk mice were anaesthetized and as previously described (Teruya *et al.* 2016) injected subcutaneously with a single dose of 4 g/kg body weight of CE for treated groups or vehicle (dH2O) for group control (see Figure S1 for the time line of

the bioassay). The CE injections were administered to the different groups of mice in a manner that all prion infections were performed on mice with same age.

All groups of mice were inoculated with 20 μL of a 1% (w/v) brain homogenate of one of the three CWD isolates (elk, MD, or WTD-116AG). Group control (Gr CTR; *n* = 5) and one of the CE-treated groups, referred to as Group A (Gr A; *n* = 10) were challenged with prions on the day of CE treatment. A third group, referred to as Group B (Gr B; *n* = 10), was challenged with prions 1 month after CE treatment (see Figure S1). The i.c. inoculation of CWD isolates was done in the right parietal lobe using a 25-gauge disposable hypodermic needle. Mice were initially monitored weekly, then daily when progressive clinical signs of prion disease were evident. At the experimental endpoint (primary endpoint), animals were exhibiting a rigid tail, rough coat, lack of balance, ataxia, hunched posture, and weight loss. They were then anesthetized, using isoflurane, before being euthanized by CO$_2$ overdose and their brains were collected and frozen at −80°C. An additional set of experiments was performed using 5 mice per group to be inoculated with 20 μL of a 1% (w/v) brain homogenate of mouse CWD-elk prions of Gr CTR (pool of 3 mice) and Gr B (pool of 3 mice) without further CE treatment. Survival was expressed as the average value of the days post inoculation (dpi).

To minimize animal suffering, Metacam [meloxicam (DIN 02240463; Boehringer Ingelheim, Canada)] was used as an analgesic. All animals in this study did receive meloxicam prior to treatment and prion inoculation. Mice received a dose of 5 mg/kg subcutaneously administered 10 min prior to any procedure and then 12 h after. Mice were re-checked for signs of pain or discomfort to determine the need for additional analgesic.

All the mice used in this study (statistical analysis and graphs) were enclosed in the inclusion criteria determined by the experimental endpoint described above. The exclusion criteria were determined based on a humane endpoint or mice, which died during the experiments of an intercurrent disease. Based on the exclusion criteria, we had to exclude mice from different groups [mCWD-elk (Gr CTR, 1 mouse), WTD-116AG (Gr CTR, 1 mouse; Gr A, 2 mice), and CWD-MD (Gr A, 1 mouse; Gr B, 1 mouse)]. None of the excluded mice were replaced in the study, therefore, no test for outliers was conducted.

PrP^{res} western blot detection

For PrP analysis in brain extracts, brain homogenates (10%) prepared in PBS from different animals were treated for 1 h at 37°C with increasing concentrations of PK (cat# 03115879001, Roche, CA, USA), from 0.025 to 5 mg/mL for TC-5RW treated groups and from 0.05 to 5 mg/mL for 60SH-50 treated groups (TC-5RW groups). The reaction was terminated by adding 1X pefabloc proteinase inhibitor (cat# 11429868001, Roche). Samples were boiled in sample buffer at 100°C for 10 min and 50 μg of protein were loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5% SDS-PAGE gel), and then electrophoretically transferred to PVDF membranes (cat# 10600023, GE Healthcare Life). PVDF membranes were blocked for 1 h in PBS-Tween (0.1%) containing skim milk powder (5%) and probed using anti-PrP monoclonal antibody 4H11 (1/500) (Ertmer *et al.* 2004) over night. Washing steps were performed using PBS-Tween buffer followed by horseradish peroxidase-

conjugated goat anti-mouse IgG antibody (cat# A9917; Sigma-Aldrich, CA, USA) for 1 h at RT and developed using ECL-plus detection (cat# WBLUF0500, Millipore, CA, USA). Images were acquired on X-ray films (cat# E3018, Denville Scientific) or using a digital imaging system (Alpha Innotech, FluoriChemQ, San Leandro, CA, USA). FluoChemQ software (Alpha Innotech) was used to quantify and determine the relative values of PrP^{res} signals.

Preparation of recombinant PrP (rPrP) substrate

The mature form of mouse (aa23-231) PrP was cloned into pET41a expression vectors (EMD Biosciences) and expressed in *E. coli* Rosetta using the Express Autoinduction System (Novagen). Inclusion bodies were prepared using the Bug Buster reagent (Novagen) and solubilized in lysis buffer (guanidine-HCl 8 M (cat# G9284, Sigma-Aldrich), Na-phosphate 100 mM, Tris-HCl 10 mM, pH 8.0 (cat# T6066, Sigma-Aldrich)) for 50 min at 23°C and then centrifuged at 16 000 *g* for 5 min at 23°C. Binding, refolding, and elution using an AKTA Explorer system has been described previously (John *et al.* 2013).

RT–QuIC assay

Real-time QuIC was performed as described previously (John *et al.* 2013; Hannaoui *et al.* 2017a). Briefly, reactions were set up in assay buffer containing 20 mM sodium phosphate (pH 6.9; cat#342483; Sigma-Aldrich), 300 mM NaCl (cat#S3014; Sigma-Aldrich), 1 mM EDTA (cat# E4884; Sigma-Aldrich), 10 µM Thioflavin T (cat# T3516; Sigma-Aldrich) and 0.1 mg/mL mouse recombinant PrP (mo-rPrP) substrate. Whenever stated, RT-QuIC reactions were supplemented with different concentrations of CEs (0.1 µg/mL to 10 µg/mL) or vehicle. Ninety-eight µL from the mixture were added to the wells of a 96 well optical bottom plate (cat# 232702, Nalge Nunc International). Quadruplicate reactions were seeded each time with 2 µL of serially diluted brain homogenates, starting from 10^{-2}, from uninfected TgElk mice, CWD isolates (elk, MD, 116AG) or brain homogenates of inoculated TgElk mice. Brain homogenates (seeds) were 10-fold serially diluted in RT-QuIC seed dilution buffer (0.05% (w/v) SDS in 1X PBS (cat# AM6925; LifeTechnologies). The plate was sealed with Nunc Amplification Tape (Nalge Nunc International) and placed in a BMG Labtech FLUOstar Omega fluorescence plate reader that was pre-heated to 42°C for a total of 50 h with cycles of 1-min double orbital shaking (700 rpm) incubation and 1-min resting throughout the incubation. ThT fluorescence signals of each well were read and documented every 15 min then the values were plotted as the average of quadruplicate reactions by using GraphPad Prism (version 7) software.

Vacuolation scoring

Brain tissues from at least two TgElk mice per inoculum group were formalin fixed (cat# BDH0502-4PL; BDH chemicals) and paraffin embedded for pathological analysis. Sagittal brain sections (5 µm-thick) were cut and stained using hematoxylin (cat# 3801571; Leica, CA, USA) and eosin (cat# 3801616; Leica) to evaluate the sections for spongiform changes. Spongiform degeneration was assessed at nine different regions of the brain (frontal cortex, basal ganglia, parietal cortex, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, and medulla/pons). Sections were scored on a scale of 0 (absence) to 5 (severe) for the presence and severity of spongiform degeneration as described previously (Fraser and Dickinson 1967;

Bruce *et al.* 1997). The scoring was performed at least six times (range 6–8) in a blinded manner. The scores are reported as the mean ± SD and the values were plotted by using GraphPad Prism (version 7) software.

Conformation stability assay (CSA) of PrP^{Sc}

CSA was performed as described previously (Hannaoui *et al.* 2017a) with slight modifications. Briefly, 10% brain homogenates from CE-treated mice (two sets of mice for each group) were incubated with increasing concentrations (0.5–4 M) of guanidine-HCl for 1 h at 20°C under gentle shaking (450 rpm). Samples were then digested using 50 µg/mL of PK for 1 h at 37°C and the reaction of PK was stopped by adding 1X pefabloc proteinase inhibitor to each sample. The samples were subjected to Western blot analysis and relative values of PrP^{res} signals were quantified as described above, using a digital imaging system (Alpha Innotech, FluoriChemQ), and plotted as a sigmoid curve using GraphPad Prism (version 7) software.

Humic acid (HA) treatment

For HA-prion incubation experiments, we used commercially available HA (cat #53680; Sigma-Aldrich). HA-prion incubation experiment was performed as described in Kuznetsova *et al.* (Kuznetsova *et al.* 2018). Briefly, identical amount of 10% brain homogenate from different group of TC-5RW treated mice inoculated with CWD-Elk prions were incubated with water (control) and increasing concentrations of HA (1 g/L, 2.5 g/L and 25 g/L) for 24 h at 4°C. Following incubation, samples were boiled in sample buffer at 100°C for 10 min and analyzed by Western blot as described above. Relative values of total PrP^{Sc} signals were quantified using a digital imaging system (Alpha Innotech, FluoriChemQ). FluoChemQ software (Alpha Innotech) was used to quantify and determine the relative values of PrP^{res} signals, which were graphed using GraphPad Prism (version 7) software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 7) software. For all transmission experiments either two-way ANOVA with Tukey's and Bonferroni's post hoc tests when there were more than two groups, or an unpaired student's *t*-test when there were two groups, were used. For experiments using immunoblots to compare CWD protease-resistant properties of different groups a two-way ANOVA with Tukey's and/or Bonferroni's post hoc tests was employed. As our data are not epidemiological data and small sample size does not allow for sample normalization, two-way ANOVA and student's *t*-test were used as they are standard ones used in this case.

Results

CEs inhibit amplification of CWD prions *in vitro*

In order to determine whether CEs exert anti-prion effects against CWD prions, we first tested CEs in prion conversion assays *in vitro*. RT-QuIC has been used to assess the anti-prion effects of numerous drugs, whether as a screening tool to discriminate various compounds or as an assay to analyze the specific effect of a given compound (Ferreira *et al.* 2014; Vieira *et al.* 2014; Schmitz *et al.* 2016; Hyeon *et al.* 2017;

Ferreira *et al.* 2018). Thus, we used RT-QuIC to assess the inhibitory effect of CEs on CWD prion seeding activity (Fig. 1). Serial dilutions (10^{-2} to 10^{-5}) of CWD-elk brain homogenates were used to seed quadruplicate RT-QuIC reactions. CE compounds TC-5RW (Fig. 1a) or 60SH-50 (Fig. 1b), at concentrations ranging from 0 μg/mL to 10 μg/mL were added to the RT-QuIC reactions. As a negative control, brain homogenate of non-prion inoculated tgElk mice was used as a seed. This permitted us to calculate the fluorescence threshold based on the average + 5 times standard deviation (5X SD) of the fluorescence values of all the negative reactions. Dilutions of which at least 50% of the replicates crossed the threshold were considered positive. RT-QuIC results of CE treated reactions showed a dose dependent decrease of the conversion efficacy when CWD-elk prions were used as seed. Regardless of the CE compound used (TC-5RW or 60SH-50), seeded RT-QuIC reactions supplemented with a concentration of 1 μg/mL of CE showed a 1000-fold lower dilution endpoint when compared to reactions without CE treatment. This inhibitory effect became more pronounced as the CE concentration increased. At a concentration of 10 μg/mL of CE, the CWD-elk seeded reactions were totally inhibited, demonstrating the strong suppressive effect of CE treatment on prion conversion activity. We have also observed in the CE treated reactions an increase in the lag phase, which is the time needed to cross the threshold.

To determine whether the inhibitory effect of CE treatment was specific to one CWD isolate or if it can target the conversion of different PrP^Sc conformers, we used two additional CWD prion isolates, WTD-116AG (Hannaoui *et al.* 2017b) and MD isolates to seed RT-QuIC reactions (Fig. 2). All reactions seeded with either WTD-116AG (Fig. 2a) or CWD-MD (Fig. 2b) prions showed similar results, comparable to CWD-elk seeded reactions treated with increasing concentration of CE TC-5RW (Fig. 1a). Regardless of the CWD prion seed used, all the reactions treated with increasing concentrations of TC-5RW showed a dose dependent decrease of seeding activity. As observed with the CWD-elk isolate, the conversion efficacy of WTD-116AG and CWD-MD was completely suppressed in RT-QuIC reactions supplemented with 10 μg/mL of TC-5RW (Fig. 2). Thus, CE treatment *in vitro* resulted in an impairment of CWD prion seeding activity in a non-strain specific manner.

CE treatment significantly extends survival of transgenic mice inoculated with CWD prions

Having seen that CEs could affect prion conversion *in vitro*, we next assessed their effects on CWD pathogenesis.

Therefore, we inoculated TgElk mice with CWD prions to test the efficacy of CE treatment *in vivo* (Figure S1). All mice were inoculated i.c. with CWD-elk prions, the mice of group control (Gr CTR; $n = 5$) did not receive CE treatment. Mice of group A (Gr A; $n = 10$) were s.c. injected with a single dose of TC-5RW on the day of prion inoculation. Mice of group B (Gr B; $n = 10$) were s.c. injected once with TC-5RW 1 month prior to i.c. prion inoculation. These experiments revealed that CE treatment extended the survival time of CWD-elk infected TgElk mice (Table 1, Fig. 3). The effects of TC-5RW on the survival of treated mice (Table 1, Fig. 3a) were statistically significant when compared to mice of Gr CTR regardless of whether the CE compound was administered 1 month prior (Gr B) or on the same day of CWD-elk infection (Gr A). There was no statistical difference when we compared the survival times of Gr A mice to those of Gr B mice. However, CE injection 1 month prior to prion inoculation (Gr B) extended more efficiently the survival time (+19%) than CE injection on the day of prion inoculation (Gr A; +12%) when compared to Gr CTR mice ($p < 0.0001$ and $p < 0.05$, respectively). Clinical presentation of CWD pathology was similar in all groups of mice.

Next, we wanted to see whether CE 60SH-50, the most effective CE compound in prion inoculated Tg7 mice (Teruya *et al.* 2016), would have similar effects. As Gr B mice treated with TC-5RW had a more extended survival time than Gr A, we decided for the 60SH-50 compound to use only two groups, group CTR and Gr B. Bioassay results using 60SH-50 applied once 1 month prior to i.c. inoculation with CWD-elk (Table 1, Fig. 4b), showed comparable efficacy to TC-5RW treatment. Survival time was extended by 20% in 60SH-50 treated mice.

To determine whether the effect of CEs against CWD prions was independent of the CWD isolate used for infection, we tested two additional CWD isolates. For this purpose, we used WTD-116AG and CWD-MD prions and CE TC-5RW treatment of tgElk mice, according to the previous scheme (Figure S1; Gr CTR, Gr A, Gr B; Table 1). Similar to TgElk mice inoculated with CWD-elk, animals from both treatment groups (Gr A and Gr B) inoculated with the WTD-116AG isolate had remarkably extended survival times of more than 30% compared to Gr CTR mice (Table 1, Fig. 4a). In addition, there was no notable difference between mice of Gr A and Gr B, leading to the conclusion that a single dose of CE treatment is equally efficient against CWD infection, whether CE treatment was administered on the same day or 1 month prior to prion infection. The effect of TC-5RW on the survival of TgElk mice inoculated with CWD-MD prions was slightly less pronounced, with 30% and 23% extension (Table 1, Fig. 4b).

Taken together, a single application of CE compounds efficiently extends the survival times of TgElk mice inoculated with prions from different cervid species, up to one third of the incubation time of non-treated mice.

CE treatment *in vivo* alters PrP^Sc PK resistance

In order to gain insights into the mode of action of CEs, we tested whether CE treatment alters the biochemical properties
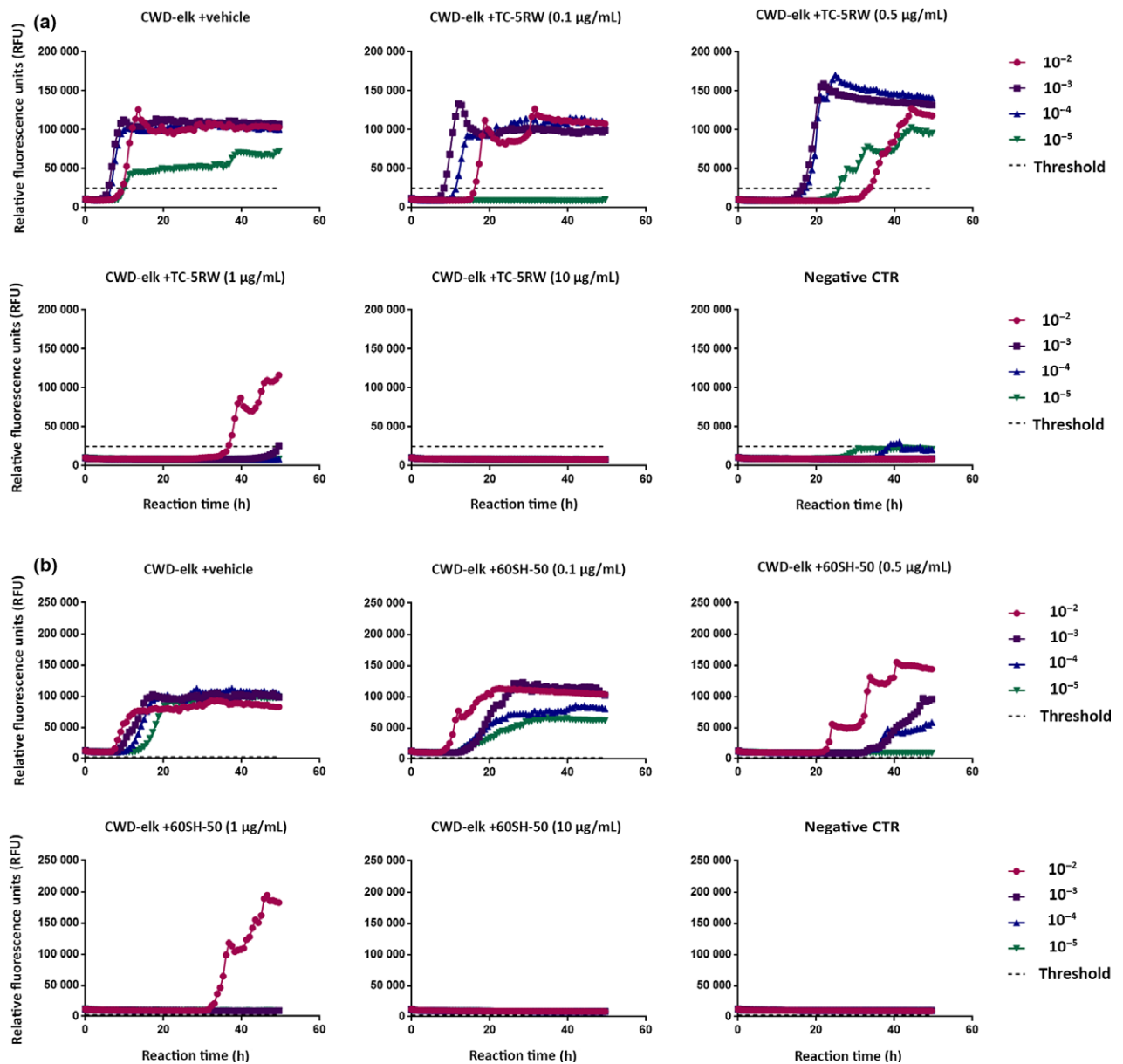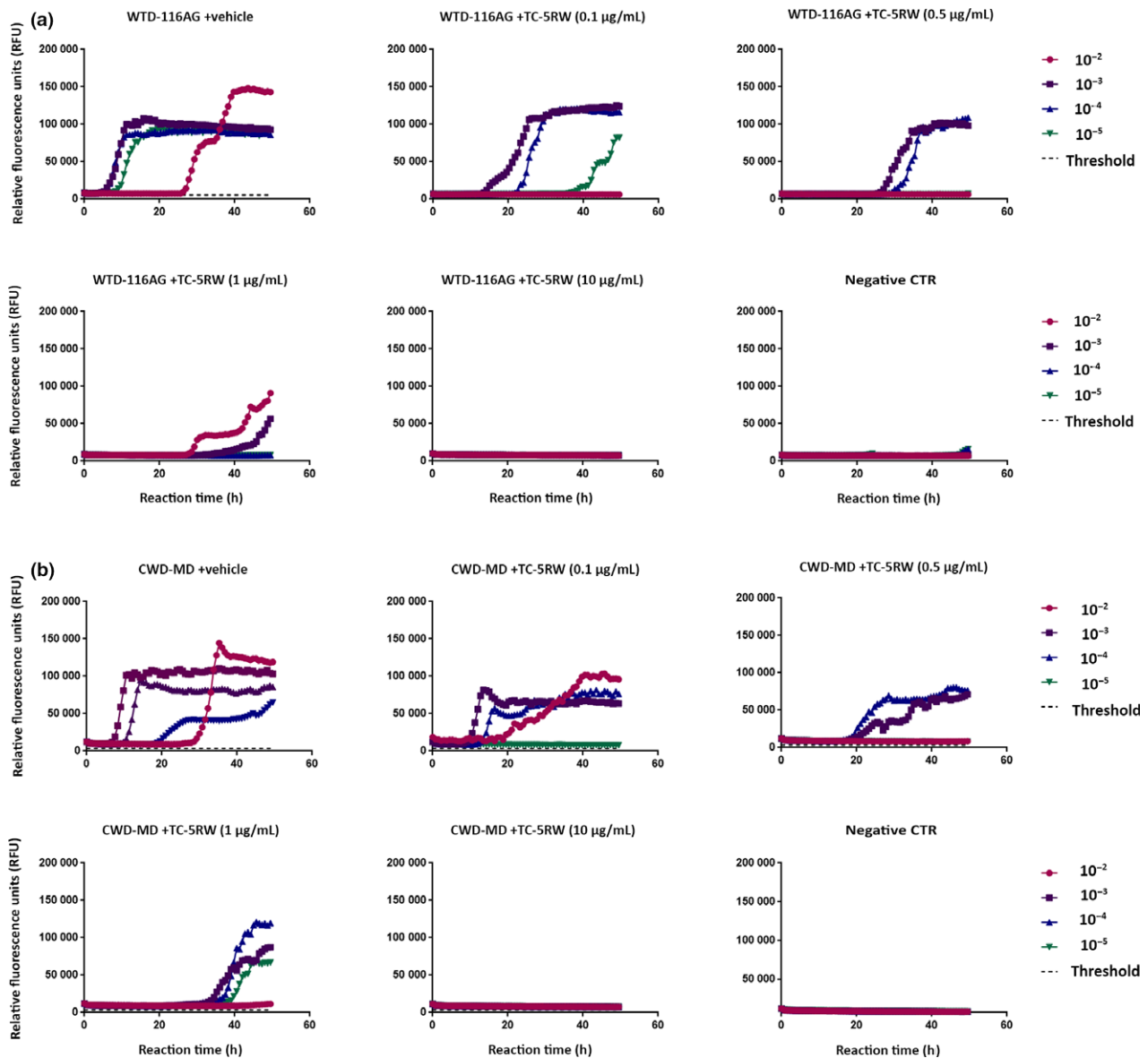
Fig. 1 The inhibitory effect of CEs on CWD-elk prion isolate seeding activity. RT-QuIC reactions were seeded with a serially-diluted CWD-elk brain homogenate (10^{-2} to 10^{-5}) using mouse rPrP as a substrate. Negative control was a naïve tgElk mouse brain homogenate. CE compounds (a) TC-5RW and (b) 60SH-50 were added at concentrations of 0, 0.1, 0.5, 1, and 10 μg/mL to each respective RT-QuIC reaction. Fluorescence was measured every 15 min. The y-axis represents the relative fluorescence units (RFUs) and the x-axis the reaction time (hours). Each curve represents a different dilution and mean values of four replicates were used for each dilution. Reactions were positive when it crossed the threshold (determined by the averaging the RFUs of the negative control + 5 SD). The curves depict a representative RT-QuIC experiment ($n = 6$ experiments).

of PrP^{Sc} in the brains of treated TgElk mice; we decided to analyze the PK resistance of PrP^{Sc}. As CE treatment worked efficiently to extend the survival times of TgElk mice independent of the CWD inoculum used for infection, we analyzed brain homogenates of control and treatment groups inoculated with CWD-elk prions. We performed PK digestion of brain homogenates from the three groups of mice (Gr CTR, Gr A, and Gr B), using different concentrations of PK (0.025 to 5 mg/mL). Western blot analysis and quantification of PrP^{res} signals indicated a dose-dependent decrease of the PrP^{res} (Fig. 5a and Figure S2) in all groups. Interestingly, PrP^{Sc} from mice treated with CE (Gr A and Gr B) was significantly more sensitive to PK, starting at 0.1 mg/mL of PK, compared to untreated control mice. When we calculated

Fig. 2 The inhibitory effect of TC-5RW CE compound on seeding activity of WTD-116AG and CWD-MD prion isolates. RT-QuIC reactions were seeded with serially-diluted CWD brain homogenates (10^{-2} to 10^{-5}) from (a) WTD-116AG and (b) CWD-MD prion isolates using mouse rPrP as a substrate. The negative control was brain homogenate from a naïve tgElk mouse. The TC-5RW CE compound was added at concentrations of 0, 0.1, 0.5, 1, and 10 µg/mL to each respective RT-QuIC reaction. Fluorescence was measured every 15 min. The y-axis represents the relative fluorescence units (RFUs) and the x-axis the reaction time (hours). Each curve represents a different dilution and mean values of four replicates were used for each dilution. Reactions were positive when it crossed the threshold (determined by averaging the RFUs of the negative control + 5 SD). The curves depict a representative RT-QuIC experiment (n = 4 experiments for each isolate).

the concentration of PK needed to reduce the PrP^{res} signals by 50%, the cPK$_{50}$, we found that the cPK$_{50}$ of PrP^{Sc} from mice treated with CE TC-5RW (Gr A and Gr B) was fivefold lower compared to that of untreated Gr CTR mice (Fig. 5b).

Next, brain homogenates of TgElk mice infected with CWD-elk and treated with 60SH-50 were digested in a similar manner, with increasing concentrations of PK (from 0.05 to 5 mg/mL) prior to western blot analysis. The quantification of PK-resistant PrP signals revealed a significant decrease at PK concentrations between 1–5 mg/mL in brain homogenates of the treatment group when compared to the brain homogenates from untreated control mice (Figure S3). These data imply that CE treatment of mice changes the protease resistance of PrP^{Sc} conformers.

Alterations of PrP^{Sc} protease resistance can affect disease progression. Therefore, we decided to inoculate TgElk mice

Table 1 Disease incubation periods of the different CWD isolates in intracerebrally-challenged tgElk receiving varying CE treatments

Mouse strain	CE compound	Inoculum	Treatment	Groups	Diseased/ injected	Average survival time (dpi ± SD)	Extended survival time (%)
TgElk	TC-5RW	CWD-elk	No treatment	[a]Group CTR	5/5	93.80 ± 3.8	--
			Same day as inoculation	Group A	10/10	105.20 ± 10.1	+12.15%
			One month prior to inoculation	[b]Group B	9/9	112.50 ± 5,3	+19.93%
	--	mCWD-elk ([a]Gr CTR)	No treatment	--	4/4	90.25 ± 5.56	--
		mCWD-elk ([b]Gr B)	No treatment	--	5/5	105.80 ± 7.91	+17.22%
	60SH-50	CWD-elk	No treatment	Group CTR	5/5	98.8 ± 3.7	--
			One month prior to inoculation	Group B	9/9	118.6 ± 9.0	+20.04%
	TC-5RW	WTD-116AG	No treatment	Group CTR	4/4	119.75 ± 11.11	--
			Same day as inoculation	Group A	8/8	159.26 ± 12.64	+32.99%
			One month prior to inoculation	Group B	10/10	156.90 ± 21.71	+31.02%
	TC-5RW	CWD-MD	No treatment	Group CTR	5/5	118.8 ± 28.27	--
			Same day as inoculation	Group A	9/9	154.37 ± 8.55	+29.94%
			One month prior to inoculation	Group B	9/9	146.00 ± 34.74	+22.89%

Group CTR (Gr CTR): this group of mice was the control group, it did not take any CE treatment. Group A (Gr A): this group of mice did take the CE treatment the same day as prion inoculation. Group B (Gr B): this group of mice did take the CE treatment 1 month prior to prion inoculation. mCWD-elk ([a]Gr CTR): brain homogenate from CWD-elk-inoculated mice, not treated with CE. mCWD-elk ([b]Gr B): brain homogenate from mice treated with TC5RW 1 month prior to CWD-elk inoculation.
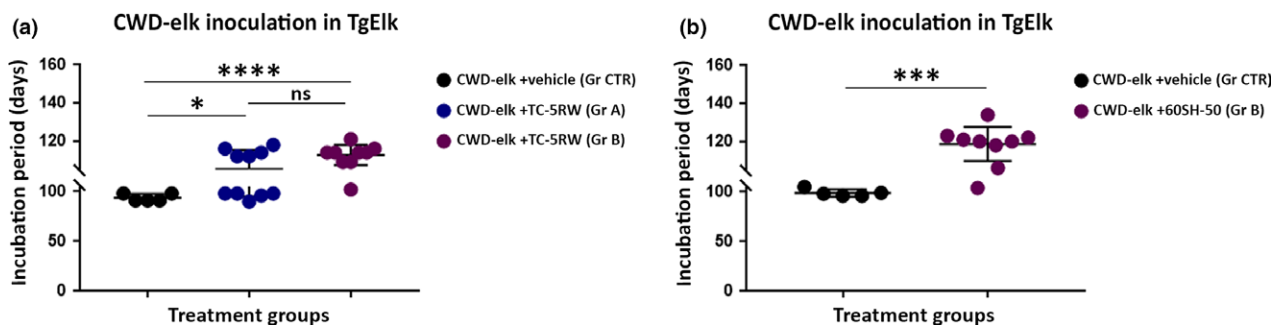
Fig. 3 Incubation periods of a CWD-elk prion isolate in tgElk mice treated with TC-5RW and 60SH-50 CE compounds. Disease incubation periods in tgElk mice treated with (a) TC-5RW and (b) 60SH-50. Mice in Gr CTR were untreated, Gr A mice were given CE on the same day as intracerebral CWD-elk prion challenge and Gr B mice were given CE 1 month prior to CWD-elk challenge. The y-axis represents the incubation period (days) and the x-axis the treatment groups. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ and ns $p > 0.05$ refers to disease incubation period differences between groups. All statistical analysis were performed using, for (a), multiple comparisons: two-way ANOVA followed by *post hoc* tests (Tukey and Bonferroni) and, for (b), an unpaired student's *t*-test in GraphPad Prism 7.0.

using pools of brain homogenates of the CWD-elk-inoculated Gr CTR and Gr B mice, respectively. Remarkably, TgElk mice inoculated with brain homogenates from Gr B mice showed an extended survival of + 17% compared to mice inoculated with Gr CTR brain homogenates (Table 1, Fig. 5c). The extended survival observed upon this passage (+17%), while no further CE treatment was applied, was very close to that upon CE treatment (+20%) when CWD-elk-inoculated mice were treated with TC-5RW compound 1 month prior to prion inoculation (Gr B). This confirmed that the altered protease resistance of PrP^Sc from CE treated mice was accompanied by a prolonged incubation period and progression of clinical disease. Furthermore, PrP^Sc of mice inoculated with brain homogenates from CE-treated mice had

a reduced PK resistance, however, only at the highest PK concentrations (Fig. 5d). Of note, the amount of seeding activity was similar in brain homogenates of control and CE treated mice (Figure S6).

Taken together, these data demonstrate that CE treatment changes PK resistance of CWD prions in the brain of treated mice. These changes were sustained but less pronounced upon passage into naïve tgElk mice.

CWD prions from CE treated mice are not resistant to CE *in vitro*

Prions can acquire drug resistance upon exposure to drugs (Ghaemmaghami *et al.* 2009; Berry *et al.* 2013; Oelschlegel and Weissmann 2013). To test whether mice treated with
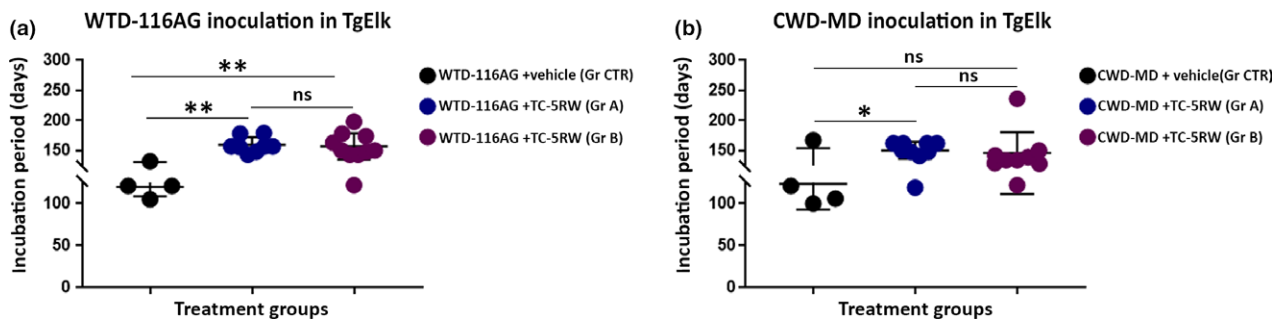
Fig. 4 Incubation periods of WTD-116AG and CWD-MD prion isolates in tgElk mice treated with TC-5RW. Disease incubation period in tgElk mice challenged with (a) WTD-116AG and (b) CWD-MD prion isolates. Mice in Gr CTR were untreated, Gr A mice were given TC-5RW on the same day as intracerebral prion challenge and Gr B mice were given TC-5RW 1 month prior to CWD-elk challenge.

The y-axis represents the incubation period (days) and the x-axis the treatment groups. *$p < 0.05$, **$p < 0.01$ and ns $p > 0.05$ refers to disease incubation period differences between groups. All statistical analysis were performed using multiple comparisons: two-way ANOVA followed by *post hoc* tests (Tukey and Bonferroni) in GraphPad Prism 7.0.

CEs and inoculated with CWD-elk generated prions resistant to the compounds, we tested brain homogenate of CWD-elk inoculated mice from treated and control groups in RT-QuIC reactions supplemented with different concentrations of TC-5RW. Accordingly, we used brain homogenates from Gr CTR (Fig. 6a), Gr A (Fig. 6b) and Gr B (Fig. 6c) mice to seed RT-QuIC reactions. TC-5RW at concentrations between 0 µg/mL to 10 µg/mL was added. Reactions without TC-5RW showed a typical RT-QuIC curve with detectable seeding activity for both tested dilutions (10^{-2} and 10^{-3}; Fig. 6). In reactions where TC-5RW was added, a dose-dependent decrease to a total inhibition of seeding activity was found for brain homogenates of control and both treatment groups (Fig. 6). The effect of TC-5RW was comparable to the dose-dependent response observed when the original CWD-elk isolate was used to seed RT-QuIC reactions (Fig. 1a).

Accordingly, we also tested brain homogenates from WTD-116AG and CWD-MD inoculated mice of different groups (Gr CTR, Gr A, and Gr B) treated with TC-5RW by RT-QuIC in presence or absence of the compound. Likewise, TC-5RW inhibited RT-QuIC reactions in a dose-dependent manner regardless of whether the seed was derived from mice of control or treatment groups, and independent of the CWD isolate (WTD-116AG or CWD-MD) used for infection (Figure S4). We also tested brain homogenates (Gr CTR and Gr B) from 60SH-50 treated TgElk mice inoculated with CWD-elk in RT-QuIC reactions supplemented with 60SH-50 or vehicle. Similarly, the results observed showed that no resistance developed against 60SH-50 CE compound (Figure S5).

Taken together, CEs effectively inhibited RT-QuIC amplification of seeds derived from both untreated and CE-treated mice, indicating that no drug resistance had developed.

In summary, these experiments reveal that a single CE treatment significantly extends survival times of transgenic mice infected with elk, MD, or WTD-116AG prions. PrP^{Sc} in the brain of treated mice has a reduced PK resistance and slower disease progression when passaged in tgElk mice, indicating changes in the biochemical properties of PrP^{Sc} conformers.

Discussion

In the past, studies showed that compounds effective against mouse prions might be inefficient against prions from other species or other prion strains (Bian *et al.* 2014). In fact, quinacrine has been shown to effectively inhibit PrP^{Sc} accumulation in cells chronically infected with mouse-adapted scrapie strains (Doh-Ura *et al.* 2000; Fasano *et al.* 2008), however, when quinacrine was tested in Elk21^{+} cells chronically infected with CWD prions to assess its efficacy against CWD prion propagation, an unexpected effect was observed (Bian *et al.* 2014). Not only did quinacrine treatment not reduce PrP^{Sc} levels, but instead it increased them in these cells. This finding was independent of the cell type (RK13 cells) used but rather was directly linked to species/strain specificity (Bian *et al.* 2014). In this well-documented case, a drug has been shown to induce conformational mutation of prions resulting in an improved replicative ability, which leads us to reflect on a wider range of species to test for each promising anti-prion compound.

CE compounds have been shown by Doh-ura's group to be very efficient against 263K hamster prions and other mouse-adapted scrapie prion strains (Teruya *et al.* 2016). Thus, we wanted to examine the efficacy of CE compounds on CWD. Here, we describe the effects of two CE compounds, TC-5RW and 60SH-50, against CWD prion propagation *in vitro* and *in vivo*. The key points of the study are that treatment with either of the two compounds was able to prolong significantly the survival of mice inoculated with one of three different CWD isolates, CWD-elk, CWD-MD, or WTD-
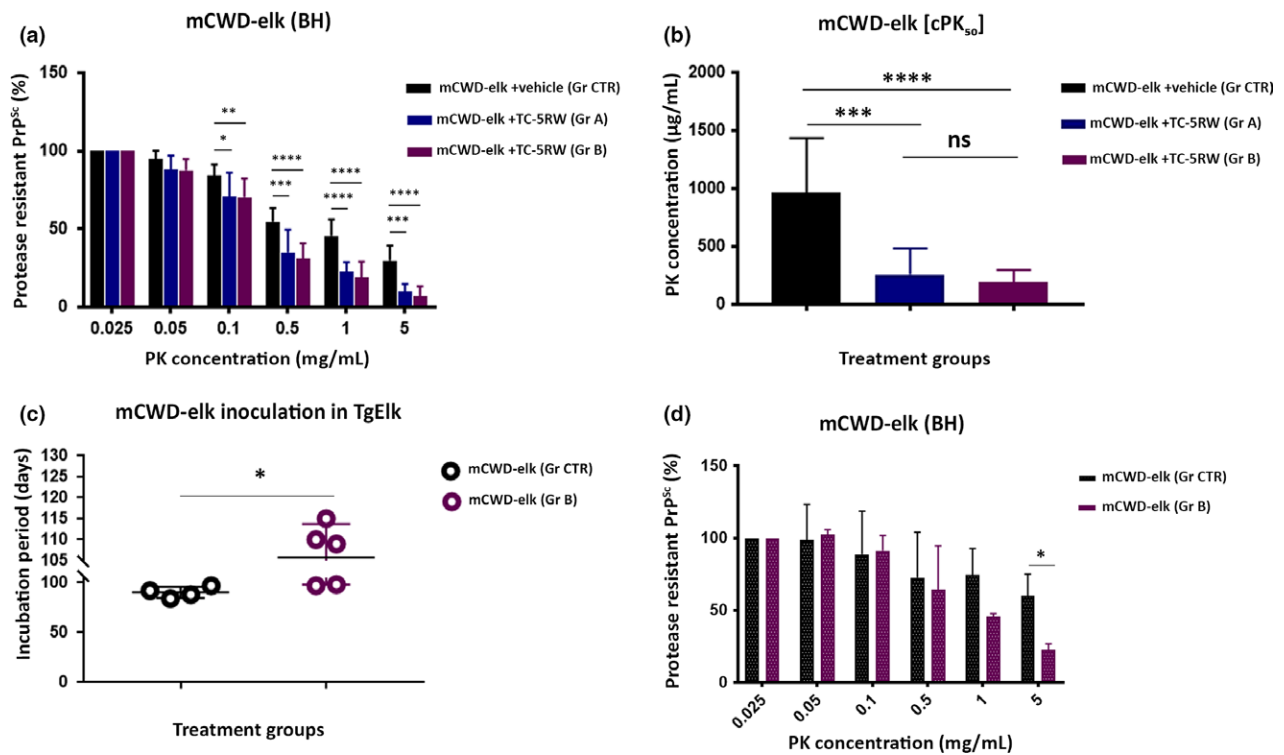
Fig. 5 Biochemical properties of protease-resistant CWD-elk prion protein (PrP^res) in brains of TC-5RW-treated tgElk mice and its incubation period upon passage. (a) PrP^res amounts in tgElk brain homogenates from the three treatment groups subjected to 0.025, 0.05, 0.1, 0.5, 1 and 5 mg/mL PK. The y-axis represents the percentage of PrP^res and the x-axis represents the different concentrations of PK. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ refers to the differences in PrP^res levels between treatment groups within one PK concentration ($n = 3$ sets of mice with 2–3 technical replicates each). Statistical analysis were performed using two-way ANOVA followed by *post hoc* (Tukey's multiple comparisons test). (b) cPK_50 of the three treatment groups. The y-axis represents the PK concentration (mg/mL) and the x-axis represents the treatment groups. *$p < 0.05$, ***$p < 0.001$ and ns $p > 0.05$ refers to the cPK_50 differences between the three treatment groups ($n = 3$ sets of mice with 2–3 technical replicates each). Statistical analysis were performed using two-way ANOVA followed by *post hoc* tests (Tukey and Bonferroni). (c) Incubation period of mCWD-elk prions in tgElk challenged i.c. Brain homogenates from Gr CTR and Gr B were i.c. inoculated again into tgElk mice. The y-axis represents the incubation period (days) and the x-axis the two groups. *$p < 0.05$ refers to disease incubation period differences between the two groups as analyzed by an un-paired student's *t*-test in GraphPad Prism 7.0. (d) PrP^res amounts in the brain homogenates of tgElk mice upon passage of Gr CTR and Gr B subjected to 0.025, 0.05, 0.1, 0.5, 1 and 5 mg/mL PK. The y-axis represents the percentage of PrP^res and the x-axis represents the different concentrations of PK. *$p < 0.05$ refers to the differences in PrP^res levels between Gr CTR and Gr B within one PK concentration ($n = 3$ sets of mice with one replicate each). Experiments using three sets of mice). Statistical analysis were performed using an unpaired student's *t* test in GraphPad Prism 7.0.

116AG. The observed extended survival was not significantly different whether the single dose of CE treatment was administered 1 month prior or at the same time of prion infection. Remarkably, concomitant to the prolonged survival of CWD-inoculated mice upon CE treatment, we showed that the resistance to PK of PrP^Sc conformers from brains of CE treated groups was reduced arguing in favor of a modification of the PrP^Sc species from PK resistant to more sensitive. Even more striking, the modified PrP^Sc species was associated with the propagation of prions inducing clinical disease with a slower progression. In fact, this was shown upon inoculation of brain homogenates from CE treated animals (Gr B) into TgElk mice, as the survival of mice inoculated with Gr B brain homogenate was prolonged

without additional CE treatment. Finally, *in vitro* results, using RT-QuIC assay, indicate that CE treatment *in vivo* did not induce drug resistance.

Various prion strains have been identified despite the absence of a nucleic acid genome, displaying specific biological properties and potentially different susceptibility to drug treatment (Telling *et al.* 1996). Therefore, we wanted to ensure that, regardless of the CWD isolate (elk, MD, or WTD) used *in vivo*, CE compounds are equally effective against CWD isolates from different cervid species (elk, MD or WTD), containing different strains (Angers *et al.* 2010; Hannaoui *et al.* 2017a). In fact, a group of tgElk mice treated with CEs and inoculated with different CWD isolates showed a significant survival prolongation of up to + 32% (Table 1).
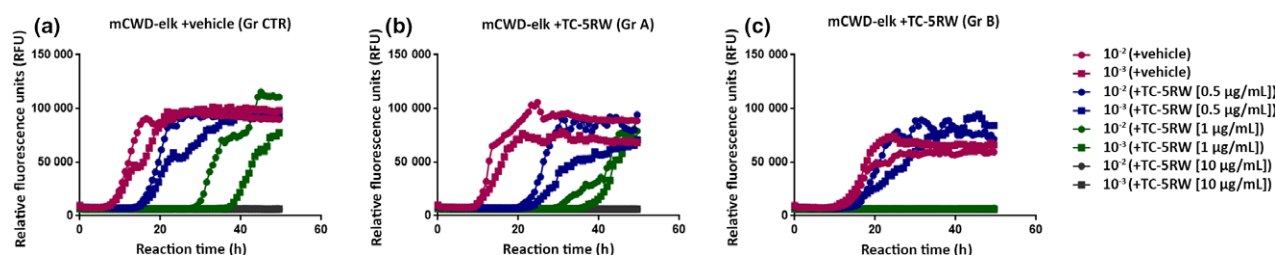
Fig. 6 The inhibitory effect of TC-5RW on CWD-elk prion seeding activity. RT-QuIC reactions were seeded with two dilutions (10^{-2} and 10^{-3}) of tgElk brain homogenates from (a) Gr CTR, (b) Gr A or (c) Gr B using mouse rPrP as a substrate. The negative control was brain homogenate from a naïve tgElk mouse. The CE compound TC-5RW was added at concentrations of 0.5, 1 and 10 µg/mL to each respective RT-QuIC dilution and group. Fluorescence was measured every 15 min. The y-axis represents the relative fluorescence units (RFUs) and the x-axis the reaction time (hours). Each curve represents a different dilution and mean values of four replicates were used for each dilution. Reactions were positive when it crossed the threshold (determined by the averaging the RFUs of the negative control + 5 SD). The curves depict a representative RT-QuIC experiment (n = 3 sets of mice with 2 technical replicates each).

This prolongation was seen with both CE compounds, TC-5RW and 60SH-50 and with all CWD isolates used for infection (Table 1), corroborating CEs as a good candidate to interfere with CWD pathogenesis and constrain CWD prion spreading regardless of the CWD strain encountered. It was not a foregone conclusion that CEs would be effective against all CWD prions tested, indeed, studies showed in the past that an anti-prion compound can be very efficient in a strain-specific manner (Cronier *et al.* 2007) even within the same species (Hannaoui *et al.* 2014).

The prolonged survival time observed in this study could be because of restrained conversion of PrP^C to PrP^{Sc} in the brains of CE treated and CWD-inoculated mice. This is supported by the results of the RT-QuIC assay (Fig. 1 and Fig. 2) where using 10 µg/mL of CEs totally inhibited the seeding activity of different CWD seeds, thus, reflecting an inhibition of PrP conversion. Ultimately, it required the same concentration of CE (10 µg/mL) to inhibit PrP^{Sc} formation whether we used here RT-QuIC or, protein misfolding cyclic amplification assay as shown previously (Teruya *et al.* 2016). Given the fact that RT-QuIC assay limits the range of possible interactions mainly to rPrP with PrP^{Sc}, the action panel of an anti-prion compound is narrowed to its capability to affect prion conversion. This latter may indicate a direct action of CE compounds on PrP^C to PrP^{Sc} conversion i*n vitro* and/or act on the seed formation, reflected by a delayed lag phase in RT-QuIC reactions supplemented with CE. Once sufficient seeds are formed to cross the threshold, the fibrils grow at similar rates along the log phase specifically in lower CE concentrations (Fig. 1 and Fig. 2).

Interestingly, treatment of brain homogenates from different groups of mice (Gr CTR, Gr A, and Gr B) with increasing PK concentration showed that PrP^{Sc} from treated mice (Gr A and Gr B) had a higher sensitivity towards PK digestion when compared to mice of Gr CTR (Fig. 5 and b; Figure S3). The increased sensitivity of PrP^{Sc} to PK digestion might be because of a reduction in particle size of PrP aggregates (Tzaban *et al.* 2002; Pastrana *et al.* 2006; Sajnani *et al.*

2012). This led us to consider whether these particles might be prone to faster clearance of PrP^{Sc} by an effective degradation process, or might promote prion propagation because of the presence of smaller and more easily converting seeds. In fact, this may explain why the effect of CE is not as efficient *in vivo* as it is *in vitro* and treated animals still get to a terminal stage of the disease. An equilibrium between these two processes can operate so that there is still propagation leading to death counterbalanced by an enhanced clearance in combination with reduced conversion rate that actually delays pathogenesis.

Even more impressively, inoculation of brain homogenates from mice of Gr B to TgElk mice revealed a significantly extended survival time when compared to mice inoculated with brain homogenates of Gr CTR. It is important here to emphasize that none of the mice upon passage did receive further CE treatment. This means that not only PK resistance of PrP^{Sc} was modified by the CE treatment and that this modification was retained, even though less pronounced, upon passage (Fig. 5d), but also the biological properties of prions were changed to some extent resulting in a prolongation of survival upon passage (Fig. 5c). RT-QuIC data showed that brain homogenates from both non-treated and CE-treated mice were carrying PrP^{Sc} with a similar seeding capacity (Figure S6), so this is not a titer effect. Besides, previous studies had clearly shown that there is a dissociation between seeding ability and infectivity (Miller *et al.* 2011; Barron *et al.* 2016; Alibhai *et al.* 2016; Groveman *et al.* 2017), which means that the observed seeding capacity, similar in different groups of mice does not necessarily reflect that their levels of infectivity are also comparable. In the past, studies had described that the addition of cofactors could change the path of recombinant prions leading to the generation of different strains with relative infectious properties (Deleault *et al.* 2012; Fernandez-Borges *et al.* 2018). Two of the defining characteristics of prion strains are conformational stability (Safar *et al.* 1998) and selective neurotropism (Bruce *et al.* 1997). Therefore, we have

analyzed the conformational stability of brain homogenates from different group of mice (Gr CTR, Gr A, and Gr B) to assess a potential strain alteration, giving rise to a new strain with different properties. Guanidine HCl denaturation of brain homogenates from CE-treated group mice resulted in the same denaturation profile when compared to that of control group mice (Figure S7a). In addition, we analyzed the neuropathological profile of mice of different groups (Gr CTR, Gr A, and Gr B; Figure S7b) and mice upon passage (Gr CTR and Gr B; Figure S7c) by scoring brain regions for spongiform changes (vacuolation). The vacuolation profiles in the brain of mice from different groups were similar to each other (Figure S7b and c). Altogether, these results are strong arguments that do not favor the scenario of alteration and rise of a new strain upon CE treatment. Thus, CE treatment did likely not lead to the generation of a stable new strain with slower replication rate, which could explain the prolonged survival. Recently, it has been shown that soil humic acids (HA) degrade CWD prions and ultimately affect prion infectivity (Kuznetsova *et al.* 2018). Thus, we tested the interaction of HA with brain homogenates of different group of mice (Figure S8) to mimic environmental degradation. Upon interaction with HA, there was no difference in the elimination of CE-exposed prions compared to control prions (Figure S8), all groups had similar decline of PrP signal following incubation with different HA concentrations. This indicates that the higher PK sensitivity relative to CE-exposed prions does not ease their degradation in the environment.

Overall, CEs might have different levels of action, either controlling the conversion of PrP^C to PrP^{Sc}, and/or modifying transiently the biochemical and biological features of PrP^{Sc}, but not necessarily its seeding capacity.

Previous studies showed that IND24 treatment was efficacious against CWD prions but not against scrapie sheep prions (Berry *et al.* 2013; Berry *et al.* 2015). However, through an unknown mechanism, IND24 can lead to drug resistance and is not as efficient against all strains/species. Besides, IND24 was delivered to transgenic mice via liquid diet every day from day 1 of inoculation, until they showed neurological signs of the disease, which is intensive and might not apply realistically for treating wild and farmed cervids. In that sense, CE compounds are more promising with an observed efficacy against CWD with only a single dose of treatment. RT-QuIC data confirmed that there is no *in vitro* resistance developed through CE treatment *in vivo* (Fig. 6, Figures S4 and S5).

CWD is one of the most contagious prion diseases. Considering the very rapid spread of the disease, it is an actual threat for cervid species (Edmunds *et al.* 2016; DeVivo *et al.* 2017). As of today, CWD management is challenging and no effective therapies or vaccines are available. The zoonotic potential of CWD has not been proven; however, the emergence of CWD strains (Angers *et al.* 2010; Duque Velasquez *et al.* 2015; Herbst *et al.* 2017;

Hannaoui *et al.* 2017a) that could more easily cross the transmission barrier in addition to conflicting data in nonhuman primates (Race *et al.* 2009; Barria *et al.* 2011; Race *et al.* 2014; Davenport *et al.* 2016; Race *et al.* 2018), makes the zoonotic potential a possibility that cannot be ruled out. Limiting the rapid spread of CWD prions among cervids will reduce both the risk of strain emergence and potential transmission to humans.

Our findings demonstrate that application of CE compounds is a valuable approach against CWD. As they are safe to use, developing it as a preventive strategy to moderate CWD spreading appears to be realistic. CE treatment resulted in the propagation of slow prions with modified biochemical properties. The mechanism by which such a phenomenon occurred is still unknown; however, these slow prion conformers retained their properties upon passage in untreated mice. Therefore, prophylactic CE treatment over time could reduce the spreading of CWD and thus limit its incidence at least in captive cervid herds.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Bioassay timeline. Group control (Gr CTR) and group A (Gr A) mice were challenged with CWD prions and given, vehicle (Gr CTR) or CE treatment (Gr A) on the same day. Group B (Gr B) mice was challenged with prions one month after CE treatment. CE was given as a single subcutaneous injection (4 g/kg body weight).

Figure S2. Western blot profile of protease-resistant CWD-elk prion in brains of CE-treated mice.

Figure S3. PrP^{res} levels in brains of 60SH-50-treated mice intracerebrally challenged with CWD-elk.

Figure S4. The inhibitory effect of TC-5RW on WTD-116AG and CWD-MD prion isolates seeding activity.

Figure S5. The inhibitory effect of 60SH-50 on passaged CWD-elk prion seeding activity.

Figure S6. mCWD-elk prion seeding activity.

Figure S7. Strain characterization of CWD-elk and mCWD-elk inoculated mice.

Figure S8. Humic-acid effect on mCWD-elk prion degradation.

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