A medicinal herb Scutellaria lateriflora inhibits PrP replication in vitro and delays the onset of prion disease in mice

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INTRODUCTION
Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases which are characterized by the accumulation and deposition of a pathogenic isoform (PrPSc) of the host encoded cellular prion protein (PrPC) designated PrPSc. Both isoforms share the same amino acid sequence but differ in conformation, resistance to proteinase K (PK), and pathogenicity. PrPSc tends to oligomerize by a seeded polymerization mechanism followed by the formation of multimers and eventually of fibril structures. This aggregation can be reproduced in an analogous manner in vitro, using cell-based as well as cell-free assays. The evolved PrPSc-like isoforms – termed PrPRes – harbor similar biochemical characteristics like resistance to Proteinase K and detection by some antibodies.

Accumulation of PrPSc in the central nervous system (CNS) is accompanied by neurological dysfunctions, neuronal vacuolation, and astrocytic gliosis. Although the exact disease causing mechanism is unknown to date, there is evidence for a general neurotoxicity of these aggregates, which deteriorate synaptic function and induce oxidative stress and membrane disruption (Soto and Estrada, 2008). Prion diseases belong to the group of protein misfolding diseases like Alzheimer’s (AD), Parkinson’s (PD), and Huntington’s disease (HD), which are generally characterized by an incorrect folding process of a host encoded protein with a conformation different from its native structure. The misfolding is followed by a self-aggregation and polymerization of the protein according to a “seeding-nucleation” process (Jarrett and Lansbury, 1993).

As PrPSc formation and aggregation is the central event in prion diseases, the inhibition of oligomer formation and fibril extension as well as the enhancement of fibril degradation are major targets for the development of therapeutic strategies against TSEs. Several substances have been identified which inhibit PrPRes formation and accumulation in vitro and prolonged survival in scrapie-infected animals: Congo red (Caughey and Race, 1992), branched polyamines (Supattapone et al., 2001), porphyrins and phthalocyanines (Priola et al., 2000; Caughey et al., 2007), heparan sulfate mimetics (Adjou et al., 2003), amphotericin (Mange et al., 2000), curcumin (Caughey et al., 2003; Yang et al., 2009), and tetracyclines (De Luigi et al., 2008). However, for various reasons none of these compounds has been included in prevention and treatment regimes for humans yet. Most recently two new substance classes, benzothiazoles (Geissen et al., 2011) and diphenylpyrazoles (Leidel et al., 2011), were identified by high-throughput screening approaches that inhibit PrPRes accumulation in cell culture models and prolong incubation times in scrapie-infected mice.

Other therapeutic strategies rely on passive immunization (White et al., 2003), RNA interference (Pfeifer et al., 2006), RNA aptamers (Prosku et al., 2002), copper chelating antibiotics (Murakami-Kubo et al., 2004), or on the induction of autophagy by Lithium (Heiseke et al., 2009).

Recent studies on AD suggest that phenolic compounds like green tea epigallocatechin gallate (Rezai-Zadeh et al., 2005), herb extracts like grape seed polyphenolic extract (Wang et al., 2009; Liu et al., 2011), or medicinal herbs like Paeonia suffruticosa (Fujiwara...
et al., 2009) can be used for the treatment of neurodegenerative diseases.

The here presented study shows that the American skullcap *Scutellaria lateriflora*, a traditional medical herb in North America, exhibits strong anti-prion activity. Extracts of this plant have been traditionally used as sedative and for the treatment of insomnia, anxiety, or neuralgia (Foster, 1996). Moreover, we can show here that the crude aqueous extract inhibits prion propagation and dissolves prion aggregates in vitro. Continuous oral administration of *S. lateriflora* tea (water extract) significantly prolonged incubation times in scrapie-infected mice. Notably, the natural conversion buffer in a final volume of 20 μl. was purchased from Goldener Zweig (Buchholz, Germany). Water extracts (tea) were prepared by mixing 30 g of sliced dry herbs in 1.0 l boiling water for 30 min. After cooling the tea was administered directly to mice. For in vitro experiments tea was lyophilized to yield dried extracts. In cell-based assays the dried material was resuspended in H2O, concentrated by a factor of 10 (termend tea concentrate) and added to the cell culture medium. In cell-free conversion assay the dried extract was directly resuspended in the conversion buffer in a final volume of 20 μl.

**PROPAGATION OF SCRAPIE STRAINS**

C57Bl/6 mice were inoculated with cloned mouse scrapie strains RML intracerebrally (i.c.; 30 μl of 1% mouse brain homogenate) or intraperitoneally (i.p.; 50 μl of 1% brain 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 (and weight loss, abnormal tail tonus, hind limb paralysis), the animals were euthanized. The incubation times were calculated as the time between inoculation and death. The brains were removed and kept as following: one half of each brain was stored at −80°C, the other half was fixed in 4% neutral buffered formalin.

Pathogenic isofrom for PrP conversion reactions was purified from brains of C57Bl/6 mice i.c. inoculated with mouse scrapie strain Me7. Brains were taken at the beginning of clinical symptoms in the animals and stored at −20°C. PrPSc was purified according to Eiden et al. (2006).

**HEMATOXYLIN AND EOSIN STAINING AND IMMUNOHISTOCHEMISTRY**

Before embedding in paraffin the formalin fixed brain was cut at five different levels to reveal caudal medulla, rostral medulla, midbrain, thalamic, and frontonal slices. Lesion profile scoring was carried out on Hematoxylin and Eosin (H&E) stained sections according to standard methodology (Fraser and Dickinson, 1968). For immunohistochemistry samples were processed (with minor modifications) as described previously (Hartd et al., 2000). Three micrometers section were de-paraffinized and rehydrated. Pretreatment included an incubation step with 98% formic acid (15 min) and blocking of the endogenous peroxidase activity with 3% H2O2/methanol (30 min) followed by autolysing in citrate buffer (pH 6.0, 20 min). The monoclonal antibody SAF 84 was applied at a dilution of 1:2000 for 2 h at room temperature and detected by avidin–biotin–horseradish peroxidase (Vector Elite). Color reactions were finally developed using diaminobenzidine (DAB) substrate.

**MATERIALS AND METHODS**

**FLAVONOIDS AND TEA OF SCUTELLARIA LATERIFLORA**

Baicalein and baicalein-hydrate were purchased from Sigma-Aldrich and solubilized in DMSO. The dried herb *S. lateriflora* was purchased from Goldener Zweig (Buchholz, Germany). Water extracts (tea) were prepared by mixing 30 g of sliced dry herbs in 1.0 l boiling water for 30 min. After cooling the tea was administered directly to mice. For in vitro experiments tea was lyophilized to yield dried extracts. In cell-based assays the dried material was resuspended in H2O, concentrated by a factor of 10 (termend tea concentrate) and added to the cell culture medium. In cell-free conversion assay the dried extract was directly resuspended in the conversion buffer in a final volume of 20 μl.

**PRP CONVERSION REACTION**

Cell-free conversion studies were performed as described before (Eiden et al., 2006; Kupfer et al., 2007). For the conversion reaction 400 ng of recombinant ovine PrPSc, expressed in *E. coli*, was incubated with 200–400 ng of purified PrPSc in a conversion buffer [50 mM citrate buffer (pH 6.0), 200 mM KCl, 5 mM MgCl2, and 1.25% sarkosyl; Horiuchi et al., 2000]. Standard conversion reactions were carried out for 3 days at 37°C. Afterward, samples were incubated with PK (final concentration of 30 μg/ml) for 1 h at 37°C. PK was diluted in TN-buffer (0.15 M NaCl, 0.05 M Tris/HCl pH 7.4). The reaction was stopped with PMSF (phenyl methansulfonyl fluoride; 10 mM). Twenty micrograms of a carrier protein (thyroglobulin) was added and the samples were incubated with a fourfold volume of methanol at −20°C to precipitate the proteins. After centrifugation at 12000 g for 15 min, the proteins were pelleted and resolubilized by heating to 95°C for 5 min in SDS-PAGE loading buffer [1% (w/v) SDS, 25 mM Tris/HCl pH 7.4, 0.5% mercaptoethanol, and 0.001% bromophenol blue].

Samples were separated on SDS-polyacrylamide gels containing 16% (w/v) acrylamide/bisacrylamide, 0.375 M Tris/HCl (pH 8.8), 0.5% (w/v) SDS (sodium dodecyl sulfate), 0.06% (w/v) APS (ammonium peroxysulfate), and 0.06% (v/v) TEMED (N,N′,N″-tetramethylethylenediamine) with a 4% stacking gel [4% (w/v) acrylamide/bisacrylamide, 0.12 M Tris/HCl (pH 6.8), 0.5% (w/v) SDS, 0.2% APS, 0.2% (v/v) TEMED].

For immunoblotting proteins were transferred in a semi-dry chamber to a polyvinylidene difluoride (PVDF) membrane. Membranes were then incubated in blocking buffer (PBS-0.1% Tween 20, 5% non-fat dry milk powder) followed by incubation for 60 min with the monoclonal antibody (mab) P4 (R-Biopharm), mab SAF-70 (SPLbio), or polyclonal antibody (pab) RA 10 (Groschup et al., 1994, 1997). In contrast to mab P4, which detects ovine PrP(Cres), pab RA10 detects only murine PrP(Cre). Membranes were washed three times for 10 min with PBS containing 0.1% Tween 20 and then incubated with a secondary antibody bound to alkaline phosphatase (Goat-anti-mouse-alkaline phosphatase or goat anti-rabbit-peroxidase). After washing, the chemiluminescence substrate CDP-Star (Tropix) was applied and membranes were incubated for 5 min before the light signals were detected by avidin–biotin–horseradish peroxidase (Vector Elite). Color reactions were finally developed using diaminobenzidine (DAB) substrate.
recorded on a Versadoc Imaging System (Biorad). Visualization was carried out with the Biorad VersaDoc™quantification software Quantity One. The percentage of converted substrate was calculated as: signal volume of PrPres digested with PK/signal volume of PrP Sc without PK × 100. Conversion rates were calculated for each time point as a mean value from four independent reactions. For stripping, membranes were incubated twice for 15 min with a buffer containing 0.2 M glycine (pH 2.0) and 1% SDS.

PRPres DETECTION FROM CELL CULTURE

Two scrapie-infected mouse cell lines were used: ScN2A-cells [scrapie infected neuroblastoma-(N2A)-cells] and SMB-cells ("scrapie-mouse-brain," infected with strain 22F, a gift of TSE USA). Survival times are expressed as mean ± SD.

RESULTS

The effect of S. lateriflora tea on the PrPres formation was first analyzed in vitro using two different scrapie-infected cell lines (SMB and ScN2A-cells), which permanently accumulate PrPres. When tea concentrate was added to the culture medium, a dose dependent reduction of PrPres accumulation was observed in SMB as well as ScN2A-cells (Figure 1). Cells were cultivated in T25 flasks and incubated with increasing amounts of S. lateriflora tea for 3 days. After this time, cells were harvested and the level of PK-resistant PrPres was assessed by Western blotting. A dilution of 1:500 induced a reduction of PrPres to 58% for ScN2A (Figure 1A, lane 2) and to 64% for SMB (Figure 1B, lane 2) cells compared to the control (Figures 1A, 1B, lane 1). Dilutions of 1:50 resulted in a reduction to 33% for ScN2A-cells (Figure 1A, lane 3) and 57% for SMB-cells (Figure 1B, lane 3). About 1:20 dilutions displayed reduction to 28% for ScN2A (Figure 1A, lane 4) and 47% for SMB-cells (Figure 1B, lane 4) and reached values of 28.7% for ScN2A-cells (Figure 1A, lane 5) and 42.3% for SMB-cells (Figure 1B, lane 5). Relative inhibition by tea is depicted in Figure 1C.

To test the direct effects of tea on the conversion of PrPC to PrPres, a cell-free conversion assay was used in which a purified murine PrPSc template induced the conversion of recombinant cellular PrPC into a PK-resistant PrPres fragment (Eiden et al., 2006; Kupfer et al., 2007). Newly converted PrPres-fragments were detected by mab P4 (Figure 2A, lanes 1–2), while PrPSc aggregates were visualized by the polyclonal antibody Ra10 (Figure 2B, lanes 1–2). Dried tea extract (3, 0.3, and 0.03 mg respectively) was added to the conversion assay, inhibited PrPres formation and led to clearance of PrPres-fragments (Figure 2A, lanes 3–6). Even 0.03 mg caused a reduced PrPres formation compared to the control (Figure 2A, lanes 7–8) by 23%.

Similar results were seen in the case of dissolution of PrPSc aggregates after PK digestion (Figure 2B). In contrast to the control (Figure 2B, lanes 1–2), PrPSc aggregates were completely digested if co-incubated with 3.0 mg dried tea extract (Figure 2B, lanes 3–4), as well as with 0.3 mg (Figure 2B, lanes 5–6). About 0.03 mg dried tea extract induced a partial disaggregation of PrPSc aggregates (Figure 2B, lanes 7–8) by 40% compared to the control.

To further characterize the underlying inhibitory mechanisms, two main polyphenolic compounds of this herbal extract, the flavonoids Baicalin (baicalein 7-O-glucuronide) and its aglycone baicalein (5,6,7-trihydroxyflavone), were studied with regard to their inhibitory properties. Both components have been isolated from this herb previously (Awad et al., 2003) and were identified in this study as ingredients in the tea by LCMS–IT–TOF hybrid mass spectrometry (data not shown). In addition, a structural analog to baicalein, the flavonoid epicatechin and quercetin were analyzed.

The potentially inhibitory effects on the PrPres accumulation and conversion were assayed by a cell-based dot blot assay system (Geissen et al., 2011) and the cell-free conversion assay. Structures, assay results, and half maximal (50%) inhibitory concentrations (IC50) of both compounds are summarized in Table 1. The cell-based dot blot assay was carried out with two different scrapie-infected SMB and ScN2A-cells again. Cells were seeded

STATISTICAL ANALYSIS

Survival times were analyzed by Kaplan–Meier Survival analysis using the log-rank test to compare the curves. The Statistical analysis was done using SigmaStat statistical software (San Jose, CA, USA). Survival times are expressed as mean ± SD.
in 96 wells and incubated with decreasing concentrations of the corresponding flavonoids (1 mM, 100 and 10 μM). IC50 values of Baicalein ranged from 138 μM (SMB-cells) to 63.7 μM (ScN2A-cells). Baicalin showed significantly lower inhibitory effects compared to baicalein in SMB-cells (IC50: >1000 μM) as well as in ScN2A-cells (IC50: 239.8 μM). Two additional flavonoids with similar structure, epicatechin, and quercetin, showed also strong inhibitory effects in both cell-types: epicatechin IC50 values were 96.6 μM (ScN2A-cells) and 106.8 μM (SMB-cells) and quercetin IC50 values were 36.8 μM (ScN2A-cells) and 274.3 μM (SMB-cells; Table 1). Baicalein displayed a strong dose dependent inhibitory effects to PrPres formation (IC50: 18.48 μM) and dissolution of PrPSc aggregates (IC50: 41.56 μM) in the cell-free conversion assay, in contrast to baicalin which yielded IC50 values of >1000 μM for PrPres inhibition and 440 μM for PrPSc dissolution. Epicatechin and quercetin, although highly similar in structural to baicalein, showed no inhibitory effects in cell-free conversion with regards to PrPres formation and PrPSc disintegration.

In summary, these data demonstrate that tea of *S. lateriflora* contains two active compounds, baicalein and baicalin, which are specific inhibitors of PrPSc conversion and PrPres amplification in vitro as well as enhance the degradation of PrPSc fibrils.

In another set of experiments in vivo effects of *S. lateriflora* tea were assessed. Wild-type mice were infected either by intracerebrally (i.c.) or intraperitoneally (i.p.) inoculation with mouse scrapie strain RML and the drinking water was replaced by freshly brewed tea starting 2 weeks prior to their inoculation and for the whole lifetime of the mice. Survival times are depicted in Figure 3. Six Bl6/C57 mice were used for the treatment and compared to six intracerebrally inoculated mice which received normal drinking water. The mean life spans of the control group (Figure 5A, bold line) was 147 ± 3.1 dpi (Figure 3B) whereas the tea treated mice (Figure 5A dashed line) survived significantly longer (184 ± 26.7 dpi; Figure 3B). Three of the treated mice survived up to 60 days compared to the control mice. In the case of i.p. inoculation of RML strain, a mean prolongation of 15 days was achieved (Figures 3C,D). The mean incubation time of the control group (Figure 3C, bold line) was 191 ± 8.6 dpi (Figure 3D) whereas the tea treated mice (Figure 3C, dashed line) survived significantly longer (206 ± 8.6 dpi; Figure 3D).

Brains of the i.c. infected mice were subjected to comparative biochemical and immunohistochemical investigations. PrP immunoblot analysis revealed no differences of PrPSc banding patterns and total amounts of PrPSc in both groups. The PrPSc content
in all brains was analyzed after PK digestion and selective precipitation of PrPC \(^*\) from corresponding mouse brain homogenates with sodium phosphotungstic acid (Figure 4). No differences in PrPC \(^*\) banding patterns or PK resistances was detected in mice which eventually developed scrapie in the tea treated and control group (Figure 4, lanes 2–13). The brain lesion profiles and PrPC \(^*\) deposition patterns in the different mouse brain regions were similar for the treated and the untreated mice (Figures 5A–D).

**DISCUSSION**

The here presented study demonstrates that solubilized ingredients of *S. lateriflora* not only inhibit the scrapie fibril formation but also destabilize pre-formed PrPC \(^*\) fibrils in a concentration dependent manner. Main constituents of the herb were the polyphenolic flavonoids baicalin and baicalein, which were found at high concentrations in corresponding extracts. Both components in purified form showed dose dependent inhibitory properties and thus were the active constituents responsible for the effects on scrapie fibril regulation.

The strongest effects displayed baicalein, the main metabolite of baicalin, which inhibited PrPC \(^*\) accumulation in both cell-based and cell-free assays and promoted the destabilization of PrPC \(^*\) fibrils at low concentrations. A possible molecular mechanism is based on direct binding to the PrPC and the formation of a stable PrP–baicalein/baicalin complex, which blocks PrP conversion and/or accumulation. In the same way the destabilization of pre-existing fibrils would be caused by a specific intercalation of baicalein/baicalin into β-sheet structures of the PrPC aggregates which may lead to a forced PrPC degradation by lysosomal proteases. Similar effects by Baicalein have been described for the inhibition of α-synuclein (Zhu et al., 2004) and amyloid-β peptide (Aβ) fibrillization (Lu et al., 2011).

**Table 1 | Chemical structures of flavonoids and in vitro analyses.**

| Compound | Dot blot | IC\(_{50}\) (μM) | Cell-free conversion assay | IC\(_{50}\) (μM) |
|----------|----------|-----------------|---------------------------|----------------|
|          | ScN2A    | SMB             | Prp\(^{\text{Pr}}\)        | Prp\(^{\text{Sc}}\) |
| Baicalein|          |                 |                           | 18.48          |
|          | 0.01 μM  | 0.01 μM         | 1.0 μM control            | 41.56          |
|          | 0.1 μM   | 0.1 μM          | 1.0 μM control            | 139.0          |
|          | 0.5 μM   | 0.5 μM          | 1.0 μM control            | 63.7           |
|          | >1000    | >1000           |                           | 239.8          |
| Quercetin| 0.01 μM  | 0.01 μM         | 0.1 μM control            | n.e.           |
|          | 0.1 μM   | 0.1 μM          | 0.1 μM control            | n.e.           |
|          | 0.5 μM   | 0.5 μM          | 0.1 μM control            | n.e.           |
|          | >1000    | >1000           |                           | n.e.           |

**IC\(_{50}\),** Half maximal (50%) inhibitory concentration; n.e., not effective.
In our present study oral administration of *S. lateriflora* tea significantly prolonged incubation of mice either infected intracerebral or intraperitoneal. These effects can be explained by direct interference of baikaline to evolving PrP aggregates in the CNS. Baicaline is able to cross the blood brain barrier and ultimately reach the CNS (Tsai et al., 2002). Baicalein, on the other hand, may act as pro-drug: After oral uptake, the sugar residue is cleaved by enzymes in the intestinal tract to generate baikalein. The more lipophilic baikaline is better absorbed in the gastrointestinal tract and can then enter the brain via the blood system (Tarragó et al., 2008).

Within the CNS, the inhibition and dissolution of prion aggregates by Baicalein could protect neuronal cells from membrane disruption and subsequent neuronal cell death. Additional antioxidant properties of baikalein on reactive oxygen species (ROS), which evolve during protein aggregation, may reinforce this
neuroprotective effect (Li et al., 2010). Baicalein is able to minimize ROS by directly quenching free radicals or indirectly inducing anti-oxidant enzymes like superoxide dismutase and catalase (Shieh et al., 2000; Kang et al., 2011).

Direct effects on the prion conversion were also seen in other plant derived polyphenols like curcumin which is a strong inhibitor of prion conversion replication in vitro (Caughey et al., 2003). It is in vivo efficacy, however, remains controversial: the oral administration of curcumin had no significant effect on the onset of scrapie in hamsters (Caughey et al., 2003), whereas scrapie-infected mice significantly survived longer after oral treatment (Riemer et al., 2008). Effects of other polyphenols (e.g., tannic acid, katechin, and 2′-2″-bis epigalloocatechin gallate) that interfere with PrP conversion in vitro were also studied in vivo (Kocisko et al., 2003). However, neither these substances nor mixtures of natural compounds including polyphenolic tea extract and pine bark extracts displayed any effects on incubation periods in scrapie-infected animals (Kocisko et al., 2004).

Up to now only few compounds have been reported that delay onset on prion diseases after oral treatment. This includes pravastatin (Vetrugno et al., 2009), which is involved in cholesterol biosynthesis and influences membrane structure and function, fucoidan (Doh-Ura et al., 2007), as well as amyloid dye derivatives (Kawasaki et al., 2007). However, possible drug associated side-effects have to be further evaluated. Recently, diphenyl-pyrazoles (Leidel et al., 2011) were identified as a new class of anti-prion compounds, which prolong incubation time of scrapie-infected mice even after oral application.

In conclusion, beneficial effects of S. lateriflora tea can be explained by the anti-aggregatory and potential anti-oxidative effects of its natural constituents, baicalein and baicalin. Only few studies were conducted with herbal extracts of S. lateriflora for prevention and therapy of protein misfolding diseases to date. Results shown here for prion diseases are promising also in the context of other diseases in this group.

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