Unique drug screening approach for prion diseases identifies tacrolimus and astemizole as antiprion agents

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Contributed by Charles Weissmann, March 4, 2013 (sent for review January 31, 2012)

Prion diseases such as Creutzfeldt-Jakob disease (CJD) are incurable and rapidly fatal neurodegenerative diseases. Because prion protein (PrP) is necessary for prion replication but dispensable for the host, we developed the PrP-FRET-enabled high-throughput assay (PrP-FEHTA) to screen for compounds that decrease PrP expression. We screened a collection of drugs approved for human use and identified astemizole and tacrolimus, which reduced cell-surface PrP and inhibited prion replication in neuroblastoma cells. Tacrolimus reduced total cellular PrP levels by a nontranscriptional mechanism. Astemizole stimulated autophagy, a hitherto unreported mode of action for this pharmacophore. Astemizole, but not tacrolimus, prolonged the survival time of prion-infected mice. Astemizole is used in humans to treat seasonal allergic rhinitis in a chronic setting. Given the absence of any treatment option for CJD patients and the favorable drug characteristics of astemizole, including its ability to cross the blood–brain barrier, it may be considered as therapy for CJD patients and for prophylactic use in familial prion diseases. Importantly, our results validate PrP-FEHTA as a method to identify antiprion compounds and, more generally, FEHTA as a unique drug discovery platform.
approximately 3–6 h (33). Removal of PrP from the cell surface by phosphatidylinositol-specific phospholipase C (PIPLC) treatment is sufficient to cure prion-infected cells (34).

Therefore, we aimed to screen for small molecules reducing cell surface PrP. Current cellular protein detection techniques such as direct or indirect immunofluorescence, or ELISA, require washing off unbound antibody. To develop a homogeneous assay obviating the need for washes and compatible with high-throughput screening (HTS) platforms, we adapted FRET to the quantification of a cellular protein. Two antibodies recognizing different epitopes of the protein of interest were added simultaneously. Double antibody occupancy generated a FRET signal proportional to the protein level, obviating the need to remove unbound antibodies. We called our HTS-ready assay the PrP-FRET-enabled high throughput assay (PrP-FEHTA). Moreover, this approach does not require prion-infected cells, thereby enabling screening of hundreds of thousands of compounds on ultra-HTS screening platforms. Using PrP-FEHTA, we performed a pilot screen by using a small collection of drugs approved for clinical use. This study led to the discovery of antiprion properties of the drugs tacrolimus and astemizole. Both inhibit prion replication in cell cultures. Moreover, astemizole, currently used to treat allergy, prolonged the survival time of prion-infected mice, thereby constituting a candidate for the treatment of human prion diseases.

Results

Screening and Hit Validation. We adapted FRET to HTS quantification of a cell surface protein. To this purpose, instead of using two labeled antibodies directed against two different proteins, which is a traditional FRET setting allowing detection of protein–protein interactions, we used two antibodies recognizing distinct domains of PrP. The assay was called PrP-FEHTA. The best signal was obtained with anti-PrP antibodies SAF32 (amino acids 53–93) and D18 (amino acids 133–157) labeled with the donor and acceptor fluorophores, respectively (Fig. S1). Because free PrP molecules shed into the culture medium by neuroblastoma cells would be detected by PrP-FEHTA leading to background signal, we used LD9 cells that exhibit minimal, if any, PrP shedding. Assay development and optimization in the 96- and 384-well formats was performed by monitoring the Z' factor, an indicator of assay robustness (defined in SI Materials and Methods) (35). The assay meets accepted performance criteria for HTS (Z' = 0.7, coefficient of variation < 10%; Fig. S2). Brefeldine A, a compound that blocks progression of proteins from the ER to the Golgi apparatus, was chosen as a pharmacological control (Fig. S2).

We screened the US Drug Collection that comprises 1,280 drugs approved for use in humans. All compounds are included in the USP Dictionary (36). The screen yielded nine hits (threshold set at 50% PrP reduction and ≤10% toxicity in the counterscreen) (Fig. 1). Interestingly, one of them was tannic acid, independently found in a screen performed by others with scrapie-infected cells (9). We selected two hits, tacrolimus and astemizole, based on their activity in orthogonal assays (cell-surface immunofluorescence and high-content quantification in neuroblastoma cells) (Fig. 2).

Tacrolimus and Astemizole Inhibit Prion Replication in Cell Culture. Our working hypothesis was that drugs reducing cell-surface PrP levels by more than 50% would significantly inhibit prion replication. We tested this hypothesis by infecting drug-treated PK1 neuroblastoma cells by either Rocky Mountain Laboratories (RML) or 22L prions and testing them for PrP\(^{\text{Sc}}\) by Western blot 9 d after infection. Treatment was stopped 12 d after infection, and cells were tested again for PrP\(^{\text{Sc}}\) 6 d later. Tacrolimus at 20 μM (i.e., the screening concentration), but not 6.7 μM, strongly inhibited the replication of both RML and 22L prions. Astemizole blocked replication of both strains at 2 μM (Fig. 3). No rebound was observed by 6 d after ending the treatment (Fig. 3).

Proposed Mechanism of Action of Tacrolimus and Astemizole. We then set out to determine the mechanism of action of the drugs. These investigations were performed at the prion inhibitory doses (PID) rather than the screening dose. Indeed, the efficacy of astemizole at 2 μM, a dose that reduced cell surface PrP by ≤20% (Fig. 2), suggested that astemizole inhibits prion propagation by a mechanism other than cell surface PrP reduction. Analysis of cell surface versus intracellular PrP by cell surface biotinylation and Western blot showed that tacrolimus treatment reduced both membrane and intracellular PrP by >70% (Fig. 4B), whereas PrP mRNA levels were unaffected (Fig. 4B). These results suggested that tacrolimus inhibits PrP translation, although accelerated PrP degradation cannot be ruled out.
surface and intracellular levels of PrP as well as PrP mRNA were not significantly affected by astemizole treatment (Fig. 4 A and B). These data show that astemizole, at the PID, inhibited prion replication by a mechanism independent of PrP expression.

Tacrolimus, also referred to as FK506, shares with rapamycin its intracellular target FKBP12 involved in calcineurin (CaN) and mammalian target of rapamycin (mTOR) signaling (37), which is key in autophagy induction. We therefore assessed whether tacrolimus induces autophagy in PK1 neuroblastoma cells. We used the conversion of the cytosolic protein LC3-I to the conjugated, autophagosome-bound form LC3-II as an indicator of autophagy induction. Astemizole was tested along with tacrolimus. Tacrolimus did not significantly modify the LC3-II/I ratio. Surprisingly, the ratio doubled after astemizole treatment, indicating autophagy induction (Fig. 5).

Collectively, these data suggest that the antiprion effect of tacrolimus is linked to a nontranscriptional regulation of PrP steady-state levels, whereas astemizole acts by enhancing autophagic function and, thereby, prion clearance (38, 39).

**Therapeutic Effect of Astemizole in Prion-Infected Mice.** Mice were intracerebrally infected with RML prions and treated from 20 to 50 d after infection (dpi) by i.p. injection of tacrolimus at 1.5 mg/kg or astemizole at 3 mg/kg. Intracerebral prion infection combined

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**Fig. 2.** Hit validation by immunofluorescence on neuroblastoma cells and high-content analysis. N2a cells were treated with DMSO 0.5% (controls) or with the drug for 24 h. (A–D) PrP staining with D18 mAb on living cells, postfixation, and epifluorescence analysis. (A and C) Controls. (B) Tacrolimus, 20 μM. (D) Astemizole, 20 μM. (E) Quantitation of the dose–response to treatment by tacrolimus or astemizole using the high-content fluorescent imaging system InCell 1000. (F) Structures of tacrolimus and astemizole.

**Fig. 3.** Tacrolimus and astemizole inhibit replication of RML and 22L prions in neuroblastoma cells without rebound after treatment cessation. PK1 neuroblastoma cells were pretreated for 3 d with the indicated doses of drugs and infected with RML (A) or 22L (B) prions. Treatment was continued for 12 d after infection (p.i.). Cells were analyzed for proteinase K-resistant PrP by Western blot 9 and 18 d p.i. (i.e., for the latter 6 d after treatment cessation). Ast, astemizole; Ctrl, vehicle-treated cells; PPS, 16 μg/mL pentosan polysulfate; Tac, tacrolimus. Drug concentrations are indicated in micromolars.

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Astemizole activates autophagy. LC3II/I ratios are elevated in astemizole, whereas astemizole has no effect on PrP levels at prion inhibitory doses (PID). PK1 cells were treated for 3 d with tacrolimus at 20 μM or astemizole at 2 μM, doses that prevented prion replication (Fig. 3). Cells were then cell surface biotinylated, and cell-surface and intracellular PrP were analyzed. Gel loading was standardized by cell number. The three blots represent three different exposure times of the same blot. (A) Tacrolimus and astemizole do not significantly alter PrP mRNA levels at PID. RT-PCR analysis of PrP mRNA levels in cells treated for 3 d with tacrolimus or astemizole at 20 and 2 μM, respectively. mRNA levels were standardized by using GAPDH mRNA.

Discussion

We have developed a drug screening approach for prion diseases with ultra-HTS capability (i.e., throughput of more than 100,000 compounds per day, the standard for automated screening platforms). To achieve this goal, we devised a strategy that does not require the handling of infectious prions. Because even partial reduction of cellular PrP levels considerably impedes prion propagation, yet is safe for the host, we developed a FRET-based HTS assay, PrP-FeHTA, for the identification of drugs that diminish the level of cell-surface PrP.

Using a 384-well PrP-FeHTA format, we screened a small collection of 1,280 Food and Drug Administration (FDA)-approved drugs to establish proof of principle and, if possible, find antiprion activity for drugs established in the clinic. After counterscreening for toxicity and hit validation in a classical immunofluorescence assay, we selected two compounds, tacrolimus and astemizole, for testing of antiprion activity in vitro. Both compounds inhibited replication in cell culture of two different prion strains, RML and 22L, chosen for their different sensitivity to other pharmacological agents (40). Because antiprion activity in cultured cells does not necessarily translate into a beneficial effect in vivo, we then tested the drug’s antiprion activity in RML-infected mice. We used a 30-d treatment regimen, which is short relative to the total infection time of ~150 d, to single out drugs with high antiprion activity in vivo. Tacrolimus showed no effect, perhaps because of its high PID of 20 μM, difficult to achieve in the brain, and/or to the short treatment duration. However, astemizole, with a PID of 2 μM, prolonged the survival of RML-infected mice. It is likely that this therapeutic effect could be improved by a longer treatment regimen and using a peripheral rather than the intracerebral route of infection.

Tacrolimus, also known as FK506, is an immunosuppressant widely used in organ transplant (41). It is also administered to ameliorate Myasthenia gravis (42). Additionally, it has been reported to slow down neurodegeneration in a murine model of prion disease (43). Tacrolimus, like rapamycin, is a canonical ligand of FK506 binding proteins (FKBPs), of which FKBP12 is the prototype. Although the rapamycin/FKBP12 complex inhibits mTOR, a controller of autophagy and cell growth, the FK506/FKBP12 complex interacts with the phosphatase CaN (37). We found that tacrolimus does not inhibit PrP transcription, yet reduces both intracellular and cell-surface PrP levels. Tacrolimus therefore regulates steady-state levels of PrP by a nontranscriptional mechanism. It remains to be determined whether tacrolimus impedes PrP translation or accelerates PrP degradation. However, it might be difficult to use tacrolimus as an antiprion drug in vivo, because of its high PID, narrow therapeutic index, and its reported neurotoxicity (44).

Conversely, we consider that astemizole may rapidly be made available to CJD patients given, on the one hand, the proof of principle of its antiprion activity in vivo and its excellent drug characteristics and, on the other hand, the rapidly fatal outcome of CJD. Astemizole is a second-generation selective histamine H₁-receptor antagonist used in humans to treat benign seasonal allergic rhinitis in a chronic setting at doses as high as 18.6 mg·m⁻² (45), corresponding to 6 mg/kg in mice, according to the assumptions and constants of Freireich et al. (46). It also possesses antifungal (47) and antimarial activity (48) and can be used for in vivo brain imaging in Alzheimer’s disease because of its affinity for Tau fibrils and good brain penetration and persistence (49). Following the launch of next generation antihistamine H₁-receptor drugs, astemizole was withdrawn voluntarily by the manufacturer in 1999 and 2003, in the United States and Europe, respectively, but generic astemizole is sold in more than 30 countries (48, 50). Astemizole underwent all pre- and postmarketing studies and has therefore an extremely well known safety and drug interaction profile. Moreover, astemizole has been an over-the-counter drug approved for pediatric and adult use for more than a decade and used for self-medication of hay fever over extended time periods (51). It has been removed from the over-the-counter market because of extremely rare occurrences of cardiac arrhythmias when overdosed (52, 53). Overall, given the suitability of astemizole for chronic administration and the extensive knowledge regarding its safe use, it seems particularly suited for prolonged preventive use, albeit under strict medical surveillance, in individuals carrying highly penetrant mutations of the PrP gene associated with familial forms of prion disease (54).

Fig. 4. Effect of tacrolimus and astemizole on PrP transcription and translation. (A) Tacrolimus reduces both intracellular and cell membrane levels of PrP, whereas astemizole has no effect on PrP levels at prion inhibitory doses (PID). PK1 cells were treated for 3 d with tacrolimus at 20 μM or astemizole at 2 μM, doses that prevented prion replication (Fig. 3). Cells were then cell surface biotinylated, and cell-surface and intracellular PrP were analyzed. Gel loading was standardized by cell number. The three blots represent three different exposure times of the same blot. (B) Tacrolimus and astemizole do not significantly alter PrP mRNA levels at PID. RT-PCR analysis of PrP mRNA levels in cells treated for 3 d with tacrolimus or astemizole at 20 and 2 μM, respectively. mRNA levels were standardized by using GAPDH mRNA.

Fig. 5. Astemizole activates autophagy. LC3II/I ratios are elevated in astemizole, but not tacrolimus-treated, cells. Cells were treated for 3 d with 20 and 2 μM of tacrolimus or astemizole, respectively. Forty or 20 μg of total proteins were loaded onto the gel.
In short, our study proposes a candidate for the treatment of prion diseases and establishes FEHTA as a unique drug discovery platform for prion and other diseases where modulation of the level of a cellular protein is beneficial. Moreover, because there is a plethora of biochemical steps whose inhibition could lead to depletion of cell surface PrP, PrP-FEHTA has the potential to identify various classes of active compounds that could be valuable probes to study PrP biosynthetic and intracellular trafficking pathways.

Last but not least, abrogation of cell surface PrP expression is potentially an innovative approach to treat Alzheimer’s disease (AD). AD is hallmarkmed by the brain deposition of plaques constituted mainly of hyperphosphorylated tau protein and amyloid β (Aβ). Inasmuch as PrP is a cell surface receptor for Aβ peptide oligomers and mediates their synaptotrophic effects (55), neuronal death (56), and memory impairment in transgenic Alzheimer’s mice (57), molecules inhibiting PrP expression at the neuronal surface may exhibit neuroprotective properties in AD.

Materials and Methods

**SI Materials and Methods** contains a detailed description of PrP-FEHTA, TR-FRET data analysis, assay performance analysis, cell viability measurements, immunocytochemistry, and high-content analysis. It also describes the methods used to analyze the compounds mode of action such as biotinylation of cell surface proteins and Western blot analysis to quantify cell surface and intracellular PrP, and RT-PCR. Details are given about animal treatments, cell treatments, cell culture, and statistical analysis.

ACKNOWLEDGMENTS.

We thank Nicole Salès for help with immunocytochemistry, Franck Madoix for advice on FRET, Patricia McDonald for support with high-content immunofluorescence analysis, and Shannon Sunday for help with animal experiments. We thank the Alafi Foundation for its generous financial support. This work was supported by the Scripps Research Institute, the Alafi Foundation, and National Institutes of Health Grant R01AG031522 (to P.H., P.C., and T.S.).

Fig. 6. Astemizole prolongs the survival time of RML prion-infected mice. Kaplan–Meier curve showing the percentages of survival of RML-inoculated mice treated from 20 to 50 d after inoculation with AST or not treated (Control). Median survival times were 149 ± 2 SEM and 155 ± 3 SEM for the control and treated group, respectively.

CJD, fatal familial insomnia, or Gerstmann–Sträussler–Scheinker syndrome.

Finally, besides astemizole’s prion inhibitory effect, we describe a hitherto unreported effect of the drug on stimulation of autophagy. Autophagy is involved in several protein misfolding neurodegenerative diseases (PMNDs) (54), and recent studies showed that enhanced autophagy counteracts cellular prion infection (38, 39). It is therefore likely that the antiprion activity of astemizole is linked to its activity on autophagy, and future studies aimed at determining the exact effect of astemizole on the autophagic pathway may reveal new therapeutic targets for prion diseases and other PMNDs.


