Systematic Identification of Antiprion Drugs by High-Throughput Screening Based on Scanning for Intensely Fluorescent Targets

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Received 23 November 2004/Accepted 7 February 2005

Conformational changes and aggregation of specific proteins are hallmarks of a number of diseases, like Alzheimer's disease, Parkinson's disease, and prion diseases. In the case of prion diseases, the prion protein (PrP), a neuronal glycoprotein, undergoes a conformational change from the normal, mainly alpha-helical conformation to a disease-associated, mainly beta-sheeted scrapie isoform (PrPSc), which forms amyloid aggregates. This conversion, which is crucial for disease progression, depends on direct PrPC/PrPSc interaction. We developed a high-throughput assay based on scanning for intensely fluorescent targets (SIFT) for the identification of drugs which interfere with this interaction at the molecular level. Screening of a library of 10,000 drug-like compounds yielded 256 primary hits, 80 of which were confirmed by dose response curves with half-maximal inhibitory effects ranging from 0.3 to 60 μM. Among these, six compounds displayed an inhibitory effect on PrPSc propagation in scrapie-infected N2a cells. Four of these candidate drugs share an N'-benzylidene-benzohydrazide core structure. Thus, the combination of high-throughput in vitro assay with the established cell culture system provides a rapid and efficient method to identify new antiprion drugs, which corroborates that interaction of PrPC and PrPsc is a crucial molecular step in the propagation of prions. Moreover, SIFT-based screening may facilitate the search for drugs against other diseases linked to protein aggregation.

Prion diseases are invariably fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD), scrapie, and bovine spongiform encephalopathy. They are caused by an unconventional infectious agent which consists primarily of the misfolded, aggregated, beta-sheet-rich PrPSc isoform of the membrane glycoprotein PrPC (29).

Scientific evidence suggests that bovine spongiform encephalopathy has been transmitted to humans, causing a new variant of CJD (4, 35), which causes major concern in regard to public health. It is unknown how many people are currently incubating the disease and will be affected by variant CJD in the future. In addition, recent evidence suggests that secondary incubation period and even after manifestation of clinical signs of disease, which is essential in addressing human prion disease.

There are a number of compounds which have been shown to be effective in interfering with PrPSc amplification, such as Congo red (15), porphyrins/phthalocyanines (7, 27, 28), Cp-60 (24), polycationic lipids (39), chemical chaperones (33), suramine (13), acridine derivatives (9, 20, 23), and variants of PrP (8). However, these compounds have been identified mostly through empirical and sometimes serendipitous observations.

Fluorescence correlation spectroscopy (FCS) allows highly sensitive analysis of protein aggregation in neurodegenerative diseases such as prion diseases at the molecular level (2, 10, 12, 19). Moreover, FCS lends itself to miniaturization and automation and has become an established method for high-throughput screening in the pharmaceutical industry (19). FCS analyzes the signal fluctuations caused by the diffusion of single fluorescently labeled molecules through an open volume element defined by the focus of an excitation laser beam that is confocally imaged on a single photon-counting detector (32). Based on this technology, we developed an assay suited for...
high-throughput screening which measures the inhibition of PrP\textsubscript{C} binding to aggregates of PrP\textsubscript{Sc}. We present the results of a screening of a library of 10,000 compounds, by which we identified a new class of drug-like substances with the potential for antiprion drugs. Moreover, the fact that compounds selected by molecular screening for inhibitors of PrP\textsubscript{C}-PrP\textsubscript{Sc} binding also induce PrP\textsubscript{Sc} clearance in cell culture corroborates that this interaction is essential in prion propagation.

MATERIALS AND METHODS

Compound library. The library screened contains 10,000 compounds and will be called DIVERSet1, because it covers only a part of the larger DIVERSet library (ChemBridge Corp., San Diego, CA). DIVERSet is a collection of rationally selected, diverse, drug-like small molecules. The compounds were supplied in dimethyl sulfoxide (DMSO) solution and on 96-well microtiter plates. A database of the compounds is available at http://www.chembridge.com.

Production of recombinant mouse PrP 23-231. Recombinant PrP 23-231 was produced and purified essentially as described previously (12), except that for expression, BL21(DE3) RIL Escherichia coli cells were transformed and cultured with ampicillin pT7Eh-Mini-PrP (ATCC 25273) for mouse PrP\textsubscript{C}.

Fluorescence labeling of antibodies and recombinant PrP. L42 monoclonal antibody (MAB) (r-biopharm, Darmstadt, Germany) against human PrP was labeled with Alexa 647 dye (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations. Recombinant mouse PrP 23-231 was labeled with the Alexa 488 dye (Molecular Probes, Eugene, OR) at a concentration of 15.75 nM potassium phosphate potassium phosphate buffer at pH 6.0, 0.1% Nonidet P-40 containing 150 to 300 M of activated dye (Alexa 488 carbocyanine, succinimidyl ester). Labeled protein and free dye were separated by gel filtration through Sephadex G-50 spin columns or PD10 columns (Amersham BioScience) using buffer A for elution.

“Scanning for intensely fluorescent targets” (SIFT) assay for PrP\textsubscript{C}-PrP\textsubscript{Sc} association. PrP\textsubscript{Sc} was prepared from brains of CJD patients according to a method described previously by Safar et al. (30), and aliquots of the final pellet suspended in 1× phosphate-buffered saline (PBS) plus 0.1% sarcosyl solution were diluted fivefold into buffer A (20 mM potassium phosphate buffer at pH 6.0, 0.1% Nonidet P-40) and sonicated in a water bath sonicator for 60 s. After centrifugation at 1,000 rpm for 1 min, the supernatant was diluted 100-fold in buffer A for the assay.

For the assay, DIVERSet1 compounds (approximately 10 mM in DMSO) were first diluted 10-fold into DMSO. This dilution was again diluted 10-fold into buffer A.

A mixture of labeled mouse recombinant PrP (rPrP) and labeled L42 monoclonal antibody was prepared in buffer A so that the labeled molecules were approximately equally abundant at 2 to 6 nM.

In a 20-μl assay volume, 8 μl of the rPrP/antibody mixture was mixed with 2 μl of the diluted compound before 10 μl of the diluted PrP\textsubscript{Sc} preparation was added. The sample was then incubated onto 96-well microscope slides with a coverslip (Evotech-Technologies, Hamburg, Germany) and measured using an Insight reader (Evotech-Technologies, Hamburg, Germany) for fluorescence intensity distribution analysis measurements for five times, 15 seconds each time, at excitation energies of 200 μW for the 488-nm laser and 300 μW for the 633-nm laser. Scanning parameters were set to 100-μm scan path length, 50-Hz beam scanner frequency, and 2,000-μm positioning table movement. Fluorescent light from the two fluorophores in the sample was recorded separately with single photon detectors, and incident photons were summed over time intervals of constant length (bins). We used a bin length of 40 μs and a measurement time of 15 s so that every measurement yielded 375,000 bins with various combinations of “green” and “red” photon counts. The frequency of specific combinations of “green” and “red” photon counts was recorded in a two-dimensional (2D) intensity distribution histogram (Fig. 1 C and D) as previously described (2, 10).

The fluorescence intensity data were evaluated using a 2D SIFT software module (EvoTech-Technologies, Hamburg, Germany) by summing up high-intensity bins in sectors as shown in Fig. 1. Cutoff values for bin intensities for each measurement series of 80 compounds and eight controls were adjusted manually according to the control measurements.

In order to establish a suitable positive control for the screening of the compound library, a selection of previously identified inhibitors of PrP\textsubscript{Sc} accumulation in cell culture were tested in the SIFT assay. Congo red, trypsin blue, and quinacrine showed either fluorescence quenching or autofluorescence which interfered with the assay. Astemizole, trifluoperazine, bebeerine, and amodiaquine exhibited only a weak inhibitory effect at high concentrations (>30 μM).

RESULTS AND DISCUSSION

The SIFT antiprion assay. To test the inhibitory effect of drugs on the association between PrP\textsubscript{C} and PrP\textsubscript{Sc}, we applied the SIFT technique (2), which utilizes an inverted dual-color confocal microscope setup. Samples were prepared in 96- or 384-well microtiter plates with coverslide glass bottoms. The assay mixture consisted of recombinant mouse PrP (rPrP), MAB L42 (34), which recognizes human PrP but not mouse PrP, and PrP\textsubscript{Sc} aggregates prepared from human CJD brain.

The rPrP and MAB molecules were labeled with green and red fluorophores, respectively. Binding of several rPrP and MAB molecules to the PrP\textsubscript{Sc} aggregates resulted in the formation of ternary complexes containing many red and green fluorophores (Fig. 1A). Such aggregates can be identified and analyzed by the SIFT method in two-dimensional fluorescence intensity histograms (2, 10) (Fig. 1C). Whenever an inhibitor of the association between rPrP and PrP\textsubscript{Sc} is included in the assay, the green fluorescence intensity of the ternary aggregates should decrease (Fig. 1B). The color distribution of the aggregates were then shifted towards the “red” sectors of the 2D histogram, as shown in Fig. 1D for a sample containing 17 μM DOSPA, a cationic lipid that has previously been found to inhibit PrP\textsubscript{Sc} formation in scrapie-infected mouse cells (9).
Primary SIFT screening of 10,000 compounds. The application of this assay system to a library of 10,000 diverse, drug-like compounds (ChemBridge DIVERSet1) is exemplified in Fig. 2A, which shows the results of a primary screening of one microtiter plate containing 80 compounds from the library and eight control samples. Three negative controls were without any additional compounds, three positive controls contained 17 μM DOSPA, and two controls lacked CJD rods and compounds and served to check the absence of aggregation in the antibody and rPrP mixture.

The three samples containing DOSPA showed reduced SIFT signal in those sectors which monitor signals of aggregates predominantly labeled with green rPrP. This indicates a decreased binding of rPrP to PrPSc. Because PrPSc is marked by the red antibody labels, their fluorescence still generates SIFT signal in the “red” sectors. Most of the compounds tested did not influence the distribution of the SIFT signal. However, some of the compounds displayed SIFT distributions shifted towards the DOSPA controls. These compounds were considered primary hits for potential antiprion drugs.

First, the compound library was subjected to a single round of screening. Only about 7% of regular measurements were unsuitable for the automated SIFT analysis, mostly because of intrinsic fluorescence of the tested compounds. This rather low percentage underscores the versatility and robustness of the SIFT assay. The identification of problematic measurements and compounds is facilitated by a multiparametric readout. For each sample, several fluorescence parameters, such as fluorescence distributions and mean intensity values for each color, are recorded simultaneously and give quality control parameters that facilitate automated detection of artifacts such as intrinsic fluorescence or fluorescence quenching by the screened compounds.

SIFT primary hits. For a compound to be classified as a primary hit, we analyzed the sum of bins in “green” sectors 1 to 5 and defined a cutoff value of approximately 50% of the effect of DOSPA compared to that of the untreated controls. With this definition, we obtained 256 primary hits from the library after a single round of screening with our SIFT assay.

For the automated quantification of the SIFT screening...
data, we have subsequently developed a software module which assigns primary SIFT activity values to the tested compounds.

Figure 2B shows the distribution of these activity values over the whole DIVERSet1 library. Assignment of a threshold value (here 0.5) identifies active compounds as primary hits.

**Validation of primary hits by dilution series.** The primary hits were checked for dose-dependent inhibition of PrPC-PrPSc association by performing the SIFT assay on a dilution series (0.1 to 100 \( \mu \)M) of each compound. For 80 compounds, these dose response curves confirmed their concentration-dependent inhibitory activity. Half-maximal inhibition of binding of rPrP to prion rods was observed at 50% effective concentration (EC50) values in the range of 0.3 to 60 \( \mu \)M compared to the effect of 17 \( \mu \)M DOSPA.

**Validation of hits in a cell culture model of prion diseases.** Promising compounds from our high-throughput in vitro assay were evaluated for their antiprion activity in a biological system. We used scrapie-infected ScN2a cells as an established cell culture model of prion propagation. The infected cells are characterized by the formation of detergent-insoluble and PK-resistant PrPsc and the propagation of infectious prions (3, 5, 6, 31). This system has been used to identify compounds which interfere with PrPsc propagation (13, 16, 33, 36, 39).

Mock-treated ScN2a cells are characterized by the presence of detergent-soluble PrPsc and the accumulation of detergent-insoluble PrPsc, which is present in the pellet fraction (Fig. 3A, control). After incubation with DOSPA, PrPsc is cleared from the infected cells (Fig. 3A, DOSPA). Note that PrPsc present in
the detergent-soluble fraction is unaffected by DOSPA. In the first screening, exemplified by the Western blot analysis shown in Fig. 3A, we tested all 80 compounds validated by the SIFT dilution series. In the ScN2a cell culture model, eight compounds interfered with the accumulation of PrPSc without showing any overt signs of cytotoxicity at a concentration of approximately 15 μM.

Six of the compounds active in the first round of cell culture analysis were available in sufficient amounts for further evaluation. These compounds were retested at a concentration of 10 μM. Notably, the relative amounts of detergent-soluble PrPSc and cytosolic Hsp70 were not affected, indicating that the compounds do not affect protein synthesis in general (Fig. 3B). Four compounds showed reproducible significant depletion of PrPSc and were thus selected for a further dose response analysis (Fig. 4A). For this analysis, the detergent-insoluble pellet fraction was incubated with PK prior to the Western blot analysis to specifically monitor the disappearance of detergent-insoluble and PK-resistant PrPSc. It turned out that compounds 293G02 and 313B02 induced clearance of PrPSc from the cells with an EC50 of about 2 and 6 μM, respectively (Fig. 4B).

**N’-Benzyldiene-benzohydrazides as new antiprion compounds.** Figure 5 shows the structures of compounds with activity against PrPSc propagation in the cell culture analysis. Four of these compounds (293G02, 309F02, 305E04, and 297F03) share a common N’-benzyldiene-benzohydrazide (NBB) core structure (cf. the first row of Fig. 6), whereas only 413 of the 10,000 compounds in the DIVERSet1 library contain this motif, suggesting a relevant structure-activity relationship.

Therefore, we investigated the influence of different substitutions around the NBB core structure on the activity found in the primary screening with the SIFT assay. To this aim, we defined a series of motifs with specific substitutions of the NBB core. Each of these structural motifs defines a class of substances from the DIVERSet1 library containing the respective motif. Figure 6 shows the distributions of the SIFT activities for eight such motifs occurring at different frequencies within the compound library.

The NBB class (Fig. 6, first row) contains many inactive compounds, but still, its activity distribution is shifted slightly towards antiprion activity. Regarding substitutions of the benzyl ring of the benzohydrazide core, we analyzed the influence of hydroxy groups. The addition of a hydroxy group at the ortho position (cf. Fig. 6, second row) leads to a smaller substance class with a similar mean activity. The classes of compounds with hydroxy groups in meta or in para position (rows 3 and 4) show increased proportions of actives. In particular, all four compounds with a combination of hydroxy groups in meta and para positions (row 6) exhibit activity in the SIFT assay. Moreover, two of these compounds were among those confirmed active in cell culture. Regarding the N’ position of the NBB core, we found a striking effect of naphthalenylmethylene substitutions. All compounds containing this moiety as the naphthalen-2-ylmethylene isomer displayed activity in the SIFT assay, whereas the median activity of compounds containing the naphthalen-1-ylmethylene isomer was close to zero. A significant structure-activity relationship is underscored by the fact that two compounds containing the naphthalen-2-ylmethylene...
group are found among the cell culture actives. Interestingly, the compound with the strongest cell culture activity (293G02) is characterized by the combination of both motifs associated with high activity in the SIFT assay. The evident correlation between SIFT activity and cell culture activity of these structural motifs indicates the feasibility of using data obtained during primary high-throughput screening in the SIFT assay for evaluation of structure-activity relationships. Moreover, this correlation indicates that the antiprion activity found in cell culture is indeed due to the inhibition of the interaction of PrPC and PrPSc by these compounds.

**Conclusion.** Here, we have demonstrated that an assay system based on the SIFT technique for the in vitro screening of large libraries of synthetic compounds is capable of identifying inhibitors of the aggregation processes accompanying prion diseases. Thereby it identifies new therapeutics for these diseases on the basis of their specific interference with the association between PrPC and PrPSc at the molecular level. Thus, the mode of action of these inhibitors is clearly defined and in accordance with the disease model postulated by the prion hypothesis (29). This novel assay system surpasses by far all assay systems in use for the search of antiprion drugs with respect to the degree of automation, the speed of measurement (75 seconds per sample), the amount of chemical compounds (only 200 picomoles per primary assay), as well as infectious agent (only the equivalent 0.2 mg of brain from a CJD case per assay) needed. We could optimize our primary screening to a throughput of 500 to 1,000 compounds per day within a university setting. Furthermore, the mapping of all screening data onto a centralized database and their automated analysis allowed the efficient evaluation and analysis of structure-activity relationships. This resulted in the identification of a new lead structure with favorable pharmacological features, which offers a new opportunity for antiprion drugs that interfere directly with the key molecular steps in prion propagation.

This new assay system for the detection of inhibitors of protein aggregation should also be adaptable to the search for new therapeutics for other neurodegenerative diseases that are linked to aggregation of specific proteins such as Alzheimer’s disease and Parkinson’s disease, as well as other diseases, in which multimer formation plays a crucial role in pathogenesis irrespective of the chemical nature of their components.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>SIFT prim. activ.</th>
<th>EC50</th>
<th>EC50</th>
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</thead>
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<tr>
<td>293G02</td>
<td></td>
<td>0.51</td>
<td>0.3</td>
<td>-2</td>
</tr>
<tr>
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<td></td>
<td>0.57</td>
<td>3.5</td>
<td>-6</td>
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<tr>
<td>309F02</td>
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<td>n.d.</td>
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<td>+</td>
</tr>
<tr>
<td>305E04</td>
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<td>+</td>
</tr>
<tr>
<td>297F03</td>
<td></td>
<td>0.43</td>
<td>30</td>
<td>(+)</td>
</tr>
<tr>
<td>260D06</td>
<td></td>
<td>0.39</td>
<td>n.d.</td>
<td>(+)</td>
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</tbody>
</table>

FIG. 5. Compounds with cell culture activity. The molecular structures of these substances and their activities in the three steps of the screening are shown. The first column shows the activities in the primary SIFT screening (SIFT prim. activ.). All these substances were validated in SIFT dilution series, and, where possible, EC50 values were determined. The last column combines the results of several steps in the cell culture system. Four of these compounds share an \( N^\prime \)-benzylidene-benzohydrazide core.

![Structural motif](image)

![SIFT activity](image)

**FIG. 6.** Structure-activity relationships for \( N^\prime \)-benzylidene-benzohydrazide derivatives. SIFT primary activities of eight substance classes are shown. The substance classes are characterized in that they contain the depicted structure motifs. The boxes and large vertical bars within the strongly occupied activity distributions mark the median and the quartiles; that statistic has been omitted for classes containing only a few members.
ACKNOWLEDGMENTS

We thank C. Schubert, B. Kraft, S. Walter, J. Mielke, and M. K. Schmidt for technical assistance. The help of I. Westner with the preparation of CID brain samples is gratefully acknowledged.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 596-B4, WI 2111/1, to J.T., K.F.W., and F.U.H. and SFB 596-B13 to A.G. and H.A.K.), the Bundesministerium für Bildung und Forschung (01KO0110 to J.T., K.F.W., and F.U.H. as well as 01KO0108 to T.H., P.T., A.G., and H.A.K.), and the state of Bavaria (ForPrion, MPI3 to J.T., K.F.W., and F.U.H.; LMU2 to U.B., J.B., and 01KO0108 to J.T., K.F.W., and F.U.H. as well as gemeinschaft (SFB 596-B4, WI 2111/1, to J.T., K.F.W., and F.U.H. and

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