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Abstract

We have developed a rapid, yeast-based, two-step assay to screen for antiprion drugs. The method allowed us to identify several compounds effective against budding yeast prions responsible for the [PSI+] and [URE3] phenotypes. These inhibitors include the kastellpaolitines, a new class of compounds, and two previously known molecules, phenanthridine and 6-aminophenanthridine. Two potent promoters of mammalian prion clearance in vitro, quinacrine and chlorpromazine, which share structural similarities with the kastellpaolitines, were also active in the assay. The compounds isolated here were also active in promoting mammalian prion clearance. These results validate the present method as an efficient high-throughput screening approach to identify new prion inhibitors and furthermore suggest that biochemical pathways controlling prion formation and/or maintenance are conserved from yeast to humans.

Main

According to the 'protein only' hypothesis1, prions are infectious proteins that cause spongiform encephalopathies in mammals. To date there is no effective treatment for these neurodegenerative disorders2, which are thought to be caused by nonconventional infectious agents that do not contain any nucleic acids but comprise a single protein, PrP. This endogenous protein is normally present in mammals, mainly in the central nervous system. PrP exists in two forms, a 'normal' (PrPC) and a pathological, misfolded form (PrPSc). PrPC consists mainly of α-helices and does not form aggregates. In this form, PrP is thought to fulfil its normal biochemical function but the exact nature of this function is not clear. In contrast, the pathological form, PrPSc, would mainly constitute β-sheets or β-helices3 and form large aggregates4. The infectiousness of the PrPSc conformation is a result of its ability to efficiently catalyze transformation of all endogenous PrPC into PrPSc through a 'snowballing effect'.

PrPSc, but not PrPC, is extraordinarily resistant to proteinase K. This characteristic is used in a common assay to detect PrPSc. After proteinase K treatment, the absence or presence of PrP indicates whether the protein was in its normal PrPC form, or in its insoluble, resistant and pathogenic PrPSc form. A mammalian in vitro model using murine neuroblastoma cells chronically infected by PrPSc has been developed5 to identify chemical inhibitors of prion accumulation. The presence of PrPSc is monitored by the proteinase K sensitivity assay. Because of the technical complexity of this screening method, investigations were limited to clinically used compounds known to cross the blood-brain barrier. Nevertheless several active molecules have been identified using this approach. The two most effective are quinacrine (an antimalaria agent) and chlorpromazine (an antidepressant). Although this approach is useful, it is clearly not amenable to high-throughput screening. For this reason, we developed a simple, rapid, economic and safe yeast-based method to screen for antiprion drugs.

The budding yeast, Saccharomyces cerevisiae, contains several proteins that behave like prions (reviewed in ref. 6). In the late 1960s, two unconventional phenotypes, [PSI+] and [URE3], were described in this organism7,8 and were later proposed to be a result of autocatalytic inactivation of the Sup35p and Ure2p proteins respectively9. These yeast prion proteins exhibit clear mechanistic analogies with PrP. In particular, both are capable of forming amyloid aggregates in vitro. As with PrP, 'normal' Sup35p is converted from a soluble

into an insoluble form when it comes into contact with another Sup35p protein in its prion form. Nevertheless, yeast prions clearly exhibit some differences compared with mammalian prions10. For example, yeast prions are not toxic11—and even seem to provide some evolutionary advantage to yeast cells12—whereas PrPSc invariably leads to death in mammals.

Guanidine hydrochloride (GuHCl) is the only known molecule effective against all budding yeast prions. After GuHCl treatment, Sup35p aggregates disappear and Sup35p becomes soluble. However, the converted Sup35p maintains its ability to be reconverted into the insoluble and infectious form. Although still controversial13, the GuHCl curing effect is probably a result of the inactivation of chaperone Hsp104p14,15,16. Deletion of the HSP104 gene17 or treatment with 1–5 mM of GuHCl (for at least six generations) cures the [PSI+] phenotype. All known budding yeast prions need Hsp104p to ensure their own autocatalytic inactivation but because this chaperone protein seems not to be conserved in higher eukaryotes, it does not constitute a good screening target for isolating antimammalian prion drugs.

In this study, a chemically highly diverse library of 2,500 compounds (synthetic and natural products purified from various sources by academic laboratories) was screened for the ability to cure the [PSI+] phenotype. Drugs active against the [PSI+] phenotype were then tested for their activity against the [URE3] phenotype using a secondary screen. Because of the structural and functional divergence of Sup35p and Ure2p, molecules active in both screens were likely to be active against a wide range of yeast prions. Using this two-step assay, we isolated six active compounds from the 2,500 screened. Five form a new class of molecules (kastellpaolitines), whereas the sixth is an already known molecule (phenanthridine). In addition, after comparing the level of activity and the molecular structure of the various isolated compounds (in a structure-activity approach), we synthesized several phenanthridine derivatives that were even more potent than the original molecules. Quinacrine and chlorpromazine, the most effective compounds to promote mammalian prion clearance in vitro, were also active in our yeast-based method. Conversely, two of the molecules identified with the yeast-based screen were tested and found to be efficient in promoting mammalian prion clearance in an in vitro system similar to the one described above5,18. These results

validate the approach as a method for identifying new antiprion drugs. Furthermore, they also suggest that, although mammalian prions and yeast prions exhibit clear differences, biochemical pathways controlling their formation and/or maintenance may be conserved from yeast to humans.

Results

Development of a yeast-based antiprion screening assay

The screening method proposed here is based on the principle that in [PSI+] cells, most of Sup35p, a subunit (also called eRF3) of the eukaryotic release factor, is sequestered into protein aggregates and thus unavailable to function in translation termination. As a result, [PSI+] causes a heritable change in protein synthesis fidelity because the ribosomes have an increased tendency to read through stop codons. The ade1-14 allele containing an opal stop codon in the open reading frame (ORF) of ADE1, a gene encoding an auxotrophic marker, provides a convenient colorimetric method of monitoring the Sup35p state17. When Sup35p is in its aggregated prion conformation ([PSI+] cells), the increased tendency of ribosomes to read through this opal codon allows cells to grow on adenine-deficient medium (SD-Ade) and prevents accumulation of metabolic byproducts. Because these byproducts lead to formation of red colonies on rich medium (yeast extract–peptone–dextrose, YPD), [PSI+] cells form white colonies on YPD. When Sup35p is in its normal soluble form ([psi-] cells), translation of the ade1-14 allele terminates at the opal codon preventing cells from growing on SD-Ade and leading to red colonies on YPD. This colorimetric assay was used to screen our chemical library for antiprion molecules (Fig. 1). To increase yeast cell permeability to chemical compounds, we constructed a strong [PSI+] strain (STRg6) from the 74-D694 strain17 in which the ERG6 gene was deleted19. As mentioned above, all known budding yeast prions need Hsp104p for their propagation and this chaperone protein was reported to be inhibited by 1–5 mM of GuHCl15,20. To increase the sensitivity of our screening method, a sub-effective dose of GuHCl was added to the medium. We choose a concentration of 200 µM. When a drug actively cures the [PSI+] prion, a halo of red colonies surrounds the filter where it was applied.

Active compound isolation and activity enhancement

Figure 2a shows all active molecules obtained upon screening of the chemical library. Initially, five new molecules belonging to the chemical family kastellpaolitines (KP1–5), as well as

phenanthridine, an already known compound, were isolated. The drugs show evident structural analogies. As equal quantities of each molecule were applied to the filters, the red halo diameter was presumably proportional to their antiprion activity, allowing classification of the compounds on this basis (Fig. 2b). KP4 was the least active and KP1 was the most active, giving a slightly stronger signal than phenanthridine. As we suspected that the amino group in position 11 in all the KPs played an important role, the corresponding position in phenanthridine was modified to yield 6-aminophenanthridine (6AP) which was much more active than all the molecules previously tested (Fig. 2c). We then added a chlorine group at position 8 in 6AP to produce 6-amino-8-chlorophenanthridine (6A-8CP), which again increased compound activity. Finally, we replaced the chlorine at position 8 by a trifluoromethyl group to yield 6-amino-8-trifluromethylphenanthridine (6A-8tFP), which lead to an additional increase in potency.

Synergy with GuHCl and a [URE3]-based secondary screen

We next investigated the influence of GuHCl on the curing effect of four of the most active molecules (phenanthridine, 6AP, 6A-8CP and 6A-8tFP). These compounds are less active on medium without GuHCl and more active on medium containing 500 µM GuHCl, as compared to cells grown on YPD supplemented with 200 µM GuHCl, our current assay condition (Fig. 3a). This synergy between our molecules and GuHCl suggests that the four compounds could act on a pathway distinct from the GuHCl-sensitive Hsp104p pathway but the possibility still remains that the compounds act by facilitating the effect of GuHCl. We also observed the same synergism for all the KPs (Fig. 3a and data not shown) and that the basal coloration of colonies shifted from white to pink when the concentration of GuHCl increased. A small quantity of GuHCl in the medium thus considerably enhances the sensitivity of the method, but at the same time also enhances background coloration. All the positive hits were also found to be active on ERG6 wild-type (wt) [PSI+] cells although substantially less than on the erg6Δ [PSI+] cells (Fig. 3b). This indicates that the activity of the compounds is independent from the ERG6 gene deletion and that inactivation of this gene clearly increases the sensitivity of the assay as observed for GuHCl21.

To define whether compounds isolated in this primary [PSI+]-based screen could be active on another yeast prion, we adapted a previously designed screen22 to set up a secondary screen

based on the [URE3] prion. For this purpose, the ORF of the DAL5 gene was replaced by that of the ADE2 gene in an ade2-1 strain. To increase cell permeability, the ERG6 gene was also deleted. In this strain (SB34), transcription of ADE2 thus depends on the state of Ure2p. If Ure2p is inactivated by a prion mechanism ([URE3] cells), ADE2 is actively transcribed, whereas if Ure2p is under its normal conformation ([ure3-0] cells), it is not. As a result, [URE3] cells of the SB34 strain will form white colonies whereas [ure3-0] cells will form red colonies. Because this strain still contains the ade2-1 allele, the red coloration observed could be a result of [PSI+] rather than [URE3] curing. This possibility was excluded after checking by cytoduction and mating that this strain was indeed [URE3] (see Methods). In addition, the entire coding sequence of the ade2-1 gene was deleted to yield the NT35 strain. This strain still formed white colonies, demonstrating again that it was indeed [URE3]. Phenanthridine, 6AP, 6A-8CP and 6A-8tFP are also active against [URE3] (Fig. 3c). We obtained the same results for KPs (data not shown). Because of the structural and functional divergence between Sup35p and Ure2p proteins, these last results suggest that our compounds are active against a wide range of budding yeast prions.

6AP efficiently cures [PSI+] and [URE3] prions in liquid culture

To exclude the possibility that active compounds were interfering directly with the colorimetric reporter system rather than with yeast prions, we carried out two types of experiments. First, upon inoculation on drug-free medium, cells from red colonies (sampled from around filters containing either active compounds or the positive control GuHCl) formed red colonies, whereas cells from the negative control still formed white colonies (data not shown), indicating that [PSI+] or [URE3] prions had been cured in cells of the red colonies. This conclusion was corroborated by curing experiments carried out in liquid medium. For this approach, we chose to use ERG6wt [PSI+] rather than erg6Δ [PSI+] cells because the latter grew poorly in liquid culture. 6AP alone caused partial curing of [PSI+]: cell patches were pink and a fraction of colonies (6.5%) were red whereas more than 90% were pink (Fig. 4a). Almost complete curing was observed when cells were grown in the presence of both 200 µM 6AP and 100 µM GuHCl: more than 90% of the cells gave red colonies, similar to the positive control (4 mM GuHCl). Taken together these results suggest that 6AP alone partially cures [PSI+] cells (or at least changes strain properties), whereas complete curing is observed when it is combined with a sub-effective concentration (100 µM) of GuHCl. This curing effect was

confirmed by verifying the aggregation state of Sup35p using differential centrifugation followed by western blot analysis. Both the dimethyl sulfoxide (DMSO) and the 100 µM GuHCl controls gave the same result as the negative control (untreated [PSI+] cells); the vast majority of the Sup35p protein remains in the pellet as expected for [PSI+] cells (Fig. 4b). In good agreement with the appearance of an average 6% red colonies, cells treated with 200 µM 6AP showed slightly more Sup35p in the supernatant. We checked using the same method that cells from two of these red colonies were indeed [psi-] (data not shown). In sharp contrast, cells treated with both 100 µM GuHCl and 200 µM 6AP contained a large fraction of soluble Sup35p similar to that obtained for the positive control (4 mM GuHCl) or for [psi-] cells. In addition, this experiment shows that pink colonies obtained after 200 µM 6AP treatment are [PSI+]. The behavior of cells from the pink colonies was further analyzed. This intermediate phenotype turned out to be meta-stable; on YPD medium containing 200 µM 6AP, cells formed dark pink colonies (data not shown). Once picked and streaked on YPD medium devoid of any compound, these cells gave rise to pink colonies (in good agreement with the pink colonies observed in Fig. 4a) but when restreaked on the same medium, the pink coloration disappeared. We believe that 6AP mimics a strain effect which depends on the presence of the drug and that, once removed, cells keep this property for a number of generations (about 18, that is, the number of generations necessary to obtain a colony) but then return progressively to their original state.

Finally we confirmed the curing effect of phenanthridine and 6AP on a second prion and using a different reporter system. [URE3] cells (CC34 strain23) grow on ureidosuccinic acid (USA) medium whereas [ure3-0] cells do not. By comparing the number of colonies in a minimal medium supplemented with uracil (SD + Ura) where both [URE3] and [ure3-0] cells grow and on USA medium where only [URE3] cells grow, the curing effect of phenanthridine and 6AP was estimated. After approximately thirty generations in YPD liquid medium containing 200 µM 6AP, more than 80% of the cells were [ure3-0]. This curing efficiency was increased to almost 100% when a sub-effective concentration of GuHCl (200 µM), together with 200 µM 6AP, was added, as in the case of the positive control (Table 1a). Thus 6AP alone is very efficient in curing [URE3] but this effect synergized with a sub-effective concentration of GuHCl. Similar results were obtained for phenanthridine, although the results were not as

clear, as this compound appeared quite toxic in the [URE3] strain used in the experiment (data not shown). Because it is possible that the absence of growth on USA medium does not reflect the curing of [URE3] but rather its inability to propagate, we checked the curing effect by cytoduction (see Methods). In addition, similar liquid culture experiments were performed with the NT35 strain (described above) where the appearance of red colonies is a result of [URE3] curing. After about twenty generations in YPD medium containing 200 µM 6AP, more than 65% of the cells gave rise to red colonies (Table 1b). This curing efficiency was increased to more than 90% when a sub-effective concentration of GuHCl (200 µM), together with 200 µM 6AP, was added, as in the case of the positive control (5 mM GuHCl).

In conclusion, we hypothesize that 6AP alone can partially destabilize Sup35p aggregates explaining the presence of pink colonies; in the case of [URE3], because the aggregates are probably smaller (ref. 24 and C.C., personal communication), 6AP alone is very efficient in curing the prion phenotype. In both cases, sub-effective doses of GuHCl synergized with 6AP leading to cure in almost 100% of the cells.

Yeast-based assay for screening mammalian prion inhibitors

To determine the ability of the method to isolate compounds efficient against mammalian prions, we determined if drugs active against PrPSc in mammalian systems5 were also active in our yeast-based assay. Quinacrine and chlorpromazine, the two most active drugs in mammalian systems were also active in the [PSI+] cell–based assay (Fig. 5a,b). Furthermore, as observed with the active compounds described here, quinacrine and chlorpromazine exhibited a strong synergistic effect with GuHCl. In contrast, no such effect was observed when 6AP was combined with quinacrine or chlorpromazine (data not shown). We also determined the activity in the yeast-based assay of other molecules tested with the murine neuroblastoma–based assay5. A good correlation between results obtained in both systems was found: acepromazine, which shows moderate activity in the mammalian system, also exhibited a weak activity in the yeast-based assay. Molecules inactive in the mammalian assay like carbamazepine, imipramine, haloperidol, chlorprothixene or methylene blue were also inactive in our assay (data not shown).

Quinacrine is also known to be an inhibitor of multiple-drug resistance (MDR)25. To test if its antiprion effect could be related to this mechanism, the curative effect of verapamil, a general and efficient MDR inhibitor was evaluated. Despite using a high and near toxic concentration of this drug, no curing effect could be detected, even on YPD medium containing 500 µM GuHCl (Fig. 5a,b and data not shown).

We next tested both KP1 and 6AP in a mammalian system similar to the one described5. KP1 was able to induce a substantial decrease in mammalian prion accumulation at 5 µM, a dose similar to the one used for chlorpromazine (Fig. 5c). After 7 d of treatment, 70% of the proteinase K–resistant PrPSc had disappeared (lanes 1 to 3) as compared to untreated cells (lanes 4 and 5). The same effect on PrPSc clearance was obtained with 6AP at 2 and 4 µM (data not shown).

Discussion

Taken together, our results validate the use of a yeast-based assay to isolate antimammalian prion compounds. Furthermore, our data strongly suggest that the biochemical mechanisms controlling formation and/or maintenance of prions in mammals and in budding yeast share important common features, which may have been conserved throughout evolution. Budding yeast thus constitutes an appropriate model to study the prion world and to screen for prion pharmacological inhibitors. Such molecules should be of great use in combating prion-based diseases, not only in humans, but also in animals26. Moreover, the potential interest of these molecules may not be limited to prion-based diseases and could probably be extended to a variety of other amyloid-based pathologies like Alzheimer, Parkinson and Huntington diseases. These pathologies have several features in common with prion-based diseases; all can be considered as 'protein folding diseases' and thus could share common mechanisms of control27,28,29,30.

What could be the mechanism of action of quinacrine, chlorpromazine and the compounds described here? We have determined that in both mammalian and yeast systems, cells need to be treated for at least several generations to observe prion clearance. This observation contrasts with the mechanism by which Congo red binds directly to the β-sheet structures of amyloidogenic proteins and presumably promotes prion clearance by quenching the buildup

of PrPSc (ref. 31). This compound only requires a few hours of treatment in mammalian cells5,32. This difference suggests that quinacrine, chlorpromazine and the the isolated compounds described here do not act directly on the aggregates but rather on the biochemical pathway required for prion formation and/or maintenance. It also suggests that these molecules could only be active in growing cells. For the moment it remains unclear whether the same drugs could also work on nongrowing cells (like neurons). Experiments are in progress to clarify this point by testing our compounds in vivo on diseased mammals. This limitation is also true for other screening assays using growing cells like the murine neuroblastoma–based assay described above5.

Our work currently focuses on the identification of the intracellular biochemical targets of the kastellpaolitines and of phenanthridine derivatives described here, which we expect to be conserved in mammals. Once identified, these cell components could become molecular targets for the development of a highly selective, mechanism-based, cell-free screening assay.

Methods

Yeast strains and culture media.

Yeast strains used in this study were as follows: Mat a, ade1-14, trp1-289, his3Δ200, ura3-52, leu2-3,112 (Strong strain of 74-D694 (ref. 17)), and Mat a, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura2:HIS3 (CC34 (ref. 23)). Standard yeast growth conditions and genetic manipulations were as described33. Yeast transformations were performed by the lithium acetate procedure. The erg6Δ [PSI+] strain (STRg6) was constructed by replacing the ERG6 gene in 74-D694 by PCR amplification of the TRP1 marker as described34 using primers oBM1060 (5′-CGATTTAAGTTTTACATAATTTAAAAAAACAAGAA TAAAATAATAATATAGTAGGCAGCATAAGCGGATCCC CGGGTTAATTAA-3′) and oBM1061 (5′-CTGCATATATAGGAAAATAGGTATATATCGTGCG CTTTATTTGAATCTTATTGATCTAGTGAATGAATTCGAGCT CGTTTAAAC-3′). The PCR product was designed to replace the entire coding region of the ERG6wt gene (from the ATG to the stop codon). After transformation of the yeast strain with the deletion cassette, successful gene replacement was demonstrated by analytical PCR on genomic DNA using primers oBM1030 (5′-GGTACCTCGTTCCCGTAC-3′) and oBM1063 (5′-CAGTCAGAAATCGAGTTCCA-3′). The SB34 strain was constructed by replacing the ERG6 gene in CC34 (ref. 23) by PCR amplification of

the TRP1 marker as described above and by replacing the coding region of the DAL5 gene by that of the ADE2 gene using the same PCR-based method as described previously for deletion of the ERG6 gene with primers 341 (5′-ACAACAAAACAAGGATAATCAAATAGTGTAAAAAAAA AAATTCAAGATGGATTCTAGAACAGTTGG-3′) and 342 (5′-TATATTCTTCTCTGATAACAATAATGTCAGTGTATCTCA CCACTATTATTACTTGTTTTCTAGATAAGC-3′). This gene replacement was then confirmed by growth on SD-Ade medium, by absence of growth on USA medium (as expected for a dal5Δ strain) and by analytical PCR on genomic DNA. The [URE3] status of this strain was checked by cytoduction: among 30 cytoductants, 26 were able to grow on USA medium showing that they were [URE3]. The NT35 strain was constructed by replacing the ade2-1 gene in the SB34 strain by PCR amplification of the KanMX marker as described above and by checking successful gene replacement by analytical PCR on genomic DNA. To test that [URE3] was cured in CC34 cells unable to grow on USA medium, their status was checked by cytoduction. For this purpose, after treatment of CC34 strain with 200 µM 6AP, we took cells from three different colonies from the SD+Ura plate (where both [URE3] and [ure3-0] cells grow) and verified their [URE3] status by growth on USA medium, cytoduction and mating. These cells were unable to form any colonies on USA medium and did not produce any [URE3] cells after either mating or transferring their cytoplasm in a [ure3-0] strain (data not shown), showing unambiguously that 200 µM 6AP efficiently cured [URE3].

Sup35p solubility assay.

Exponentially growing cells were harvested rapidly by centrifugation at 4 °C and the cell pellets were resuspended in lysis buffer (25 mM Tris, pH 7.4, 100 mM NaCl, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). After the addition of 425–600 µm glass beads, cells were lysed by vortexing for 30 s followed by 30 s ice-cooling (six times). After centrifugation, the total yeast proteins were collected in the supernatant. Lysates were subjected to centrifugation at 109,000g (50,000 rpm in a RP100-AT rotor, Sorvall RCM120EX ultracentrifuge) for 30 min at 4 °C. Supernatant and pellet fractions were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were incubated with primary antibody (anti-Sup35p rabbit polyclonal antipeptide against amino acids 55–68, (a kind gift from S.L. Lindquist, University of Chicago) 1:1,000 in TBST buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h, washed with TBST, incubated with

goat anti-rabbit antibody conjugated to horseradish peroxidase (1:3,000). Immune complexes were visualized with enhanced chemiluminescence.

PrPSc inhibition assay in ScN2a-22L cells.

Scrapie-infected murine neuroblastoma cells (ScN2a-22L)18 were a gift from S. Lehmann (Centre National de la Recherche Scientifique). Cells were cultured in 25 cm2 flasks in the presence or absence of compounds for 7 d. Subsequently, proteins were extracted from ScN2a-22L cells by lysing the cells in 500 µl lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.5 % Triton X-100). After protein normalization with an Uptima Interchim kit, adjusted quantities of cell lysates were digested with proteinase K at 20 µg/ml (Eurobio) for 40 min at 37 °C. The lysates were then centrifuged for 90 min at 20,000g and the pellet was resuspended in 25 µl of denaturing buffer (1X Tris-glycine, 4% SDS, 2% β-mercaptoethanol, 5% sucrose, bromophenol blue) and heated for 5 min at 100 °C before analysis by western blotting according to standard protocol using mouse monoclonal antibody anti-PrP SAF83 (SPI-BIO). Inhibition percentages of proteinase K–resistant PrPSc formation were calculated using the National Institutes of Health Image/J software; inhibition of PrPSc accumulation was 96% ± 3% for chlorpromazine and 70% ± 6% for KP1.

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Figure 1: Schematic representation of the antiprion compound screening assay.

An aliquot of an overnight culture (1) of the appropriate erg6Δ [PSI+] strain, STRg6 (which grows as white colonies), was spread on a petri plate containing YPD medium supplemented with 200 µM GuHCl, and small filters (similar to the ones used for antibiograms) were placed on the agar surface (2). Individual compounds were applied to each filter (3), except for the top left filter where DMSO was added (negative control, −) and for the bottom right filter where 5 µl of a 300 mM GuHCl solution in DMSO was added (positive control, +). When a compound was active against [PSI+], a halo of red colonies appears around the filter where it was spotted (6). In the example shown, two molecules gave a positive signal. The positive hits obtained were tested in a secondary screen based on [URE3], another yeast prion.

Figure 2: Isolation and activity enhancement of active compounds using a structure/activity approach.

figure2

(a) The same quantity (15 µl of a 10 mM solution in DMSO) of each of the molecules was filter-spotted on YPD medium supplemented with 200 µM GuHCl. The diameter of the red halos is therefore presumably proportional to the activity of each compound. (b) Molecules are classified (left to right) from least to most active. The molecular structure of each molecule is depicted. (c) 6-aminophenanthridine (6AP), 6-amino-8-chlorophenanthridine (6A-8CP) and 6-amino-8-trifluoromethylphenanthridine (6A-8tFP) were derived from phenanthridine (Phen); 5 µl of a 10 mM solution in DMSO of each compound were spotted. Note that they are much more active than phenanthridine.

Figure 3: Synergy between active compounds and GuHCl and implementation of a [URE3]-based secondary screen.

figure3

(a) Active compounds (5 µl of a 10 mM solution in DMSO) were tested against erg6Δ [PSI+] cells (STRg6) on YPD without GuHCl (top), or with 0.2 mM (middle) or 0.5 mM GuHCl (bottom). Note that increasing the quantity of GuHCl in the medium increases both the activity of the compounds (thus the sensitivity of the method) but also increases the background coloration. (b) The same experiment was carried out using ERG6wt [PSI+] cells (74-D694) grown on YPD containing 200 µM GuHCl. Note that ERG6wt cells were less sensitive to the treatments than

erg6Δ cells and that even the positive control gave a barely visible effect when the same quantity of GuHCl as in the previous experiment was spotted on the filter. (c) In the [URE3] strain used (SB34), the ADE2 gene was under the control of the DAL5 promoter so that cells formed white colonies. In contrast, [ure3-0] derivatives gave rise to red colonies. 20 µl of a 10 mM solution of phenanthridine, 6AP, 6A-8CP, 6A-8tFP in DMSO or 20 µl of a 300 mM GuHCl solution in DMSO were spotted. Phenanthridine, 6AP, 6A-8CP and 6A-8tFP are also active in this secondary screen based on [URE3] prion. Note that this secondary screen is fully functional but less sensitive than the primary one based on [PSI+].

Figure 4: 6AP efficiently cures [PSI+] prion in liquid culture.

figure4

(a) ERG6wt [PSI+] cells (74-D694) were maintained in exponential growth phase at 24 °C for 5 d (~40 generations) by diluting them every 24 h in fresh YPD liquid medium in the presence of the indicated concentration of the different molecules. Every 24 h, 104 cells were washed in compound-free medium and spotted as 15 µl drops on YPD plates (days 0–5, left row). On the right row are pictures of cell colonies obtained by streaking an aliquot fraction of each cell culture at the end of the experiment (day 5). Note that 6AP alone was partially able to cure [PSI+] cells; treatment with 200 µM of this drug resulted in 6.5% red colonies and more than 90% pink colonies. In combination with 100 µM GuHCl, 6AP was able to efficiently cure [PSI+] cells (more than 90% red colonies). (b) Sup35p solubility assay. After cell lysis, high-speed centrifugation and separation by SDS-PAGE, Sup35p was detected by immunoblotting with an anti-Sup35p antibody. S, supernatant fraction; P, pellet fraction. Note that cells treated with 200 µM 6AP in combination with 100 µM GuHCl have a large fraction of soluble Sup35p, similar to the one obtained for positive controls (cells treated with 4 mM GuHCl and [psi-] cells).

Figure 5: Validation of the yeast-based method for screening mammalian prion inhibitors.

figure5

(a) ERG6wt [PSI+] cells (74-D694) were treated as shown in Figure 1: 15 µl of 10 mM solutions of 6AP, quinacrine, chlorpromazine and verapamil were spotted. Note that both quinacrine and chlorpromazine were active though much less than 6AP. Even at a very high concentration just below the toxicity level, verapamil was inactive. (b) The same experiment

carried out with erg6Δ [PSI+] cells (STRg6). Note that quinacrine and chlorpromazine are also active with this strain. (c) KP1 was tested against mammalian prion in an in vitro system similar to the one described before5. Note that KP1 was able to induce a substantial decrease in mammalian prion accumulation (compare lanes 1–3, triplicate of the same experiment, to lanes 4–6).

Table 1 6AP effectively cures [URE3] prion in liquid culture

Figure 1 Bach et al.,

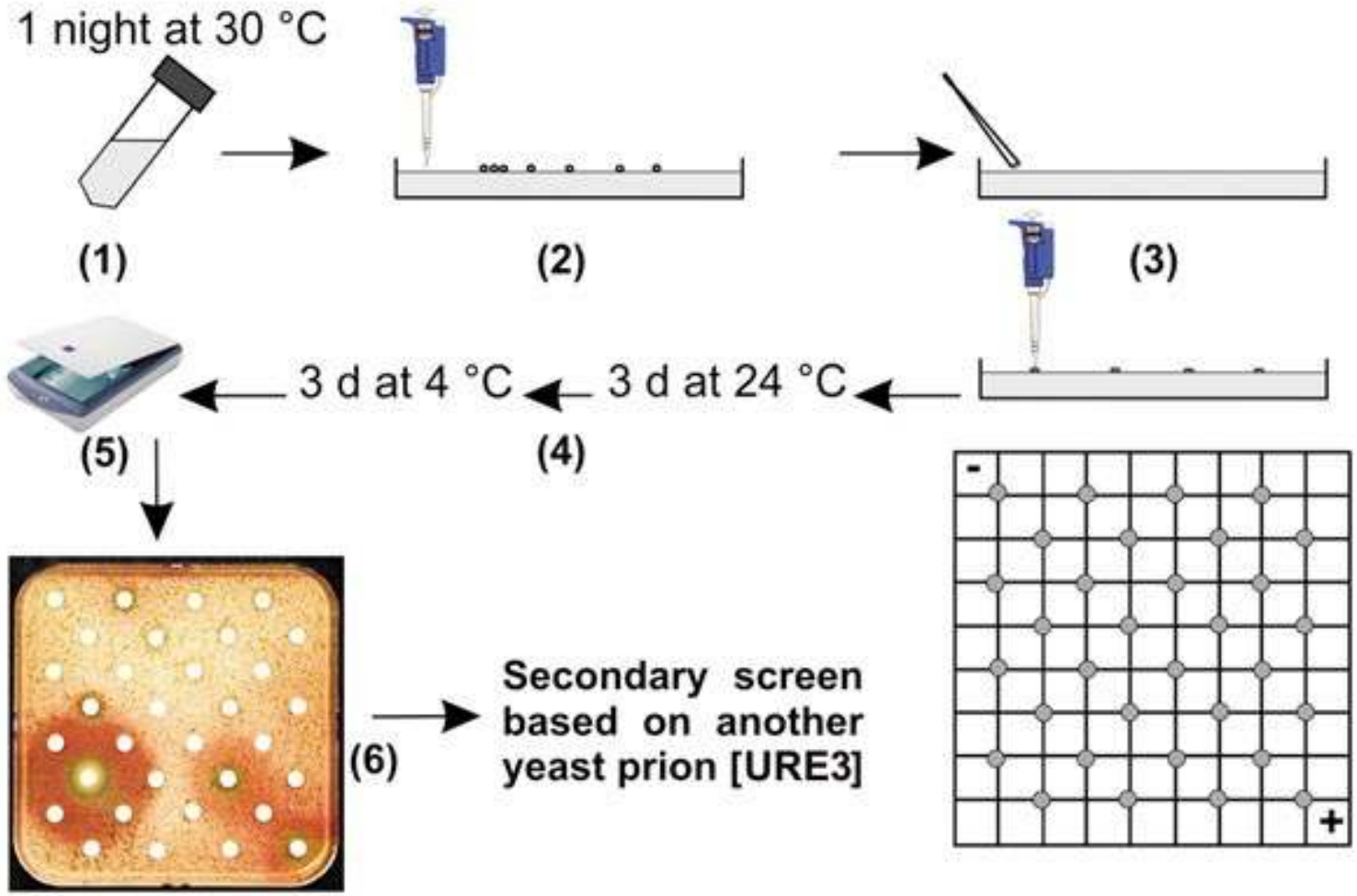


Figure 2 Bach et al.,

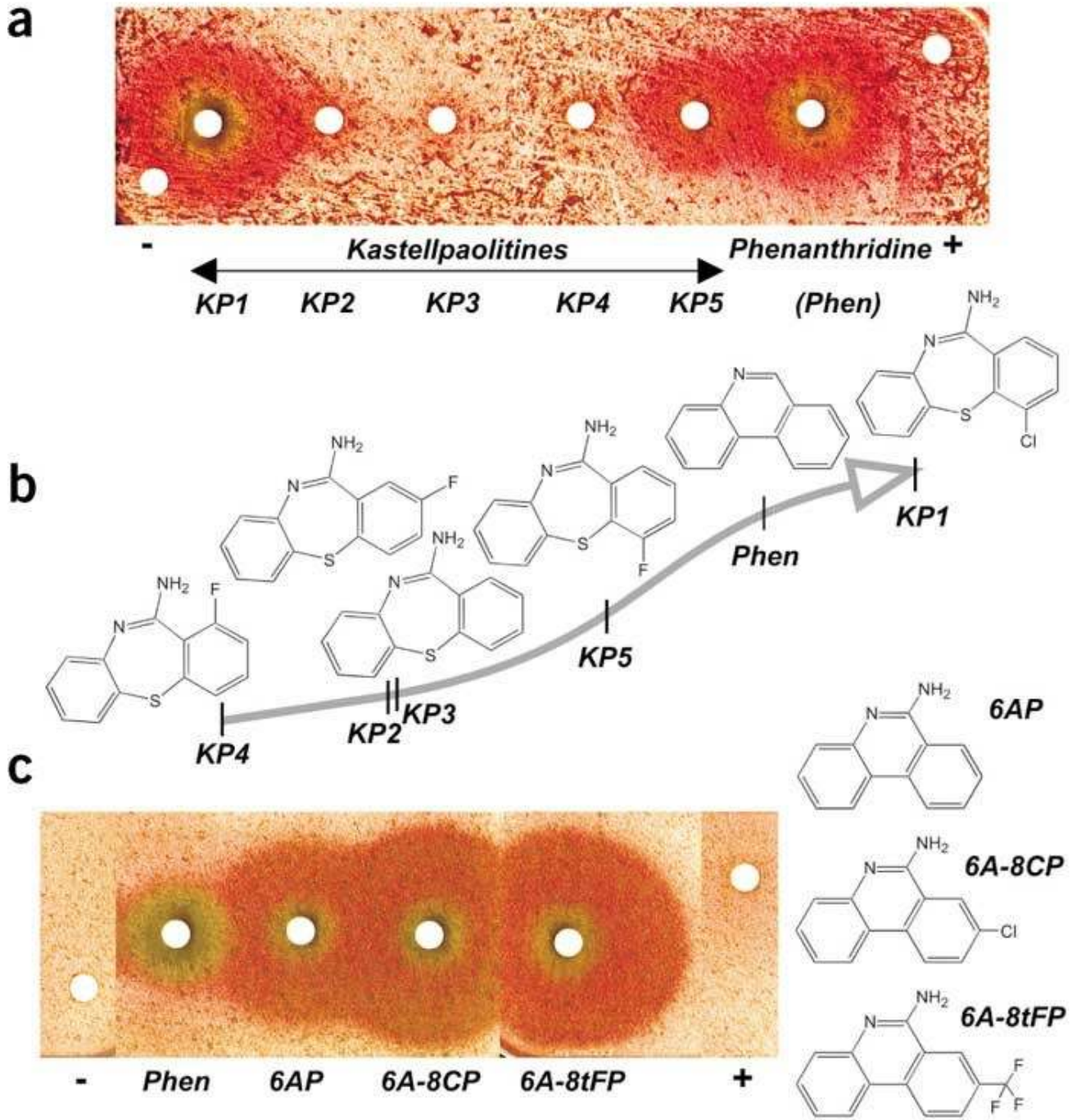


Figure 3 Bach et al.,

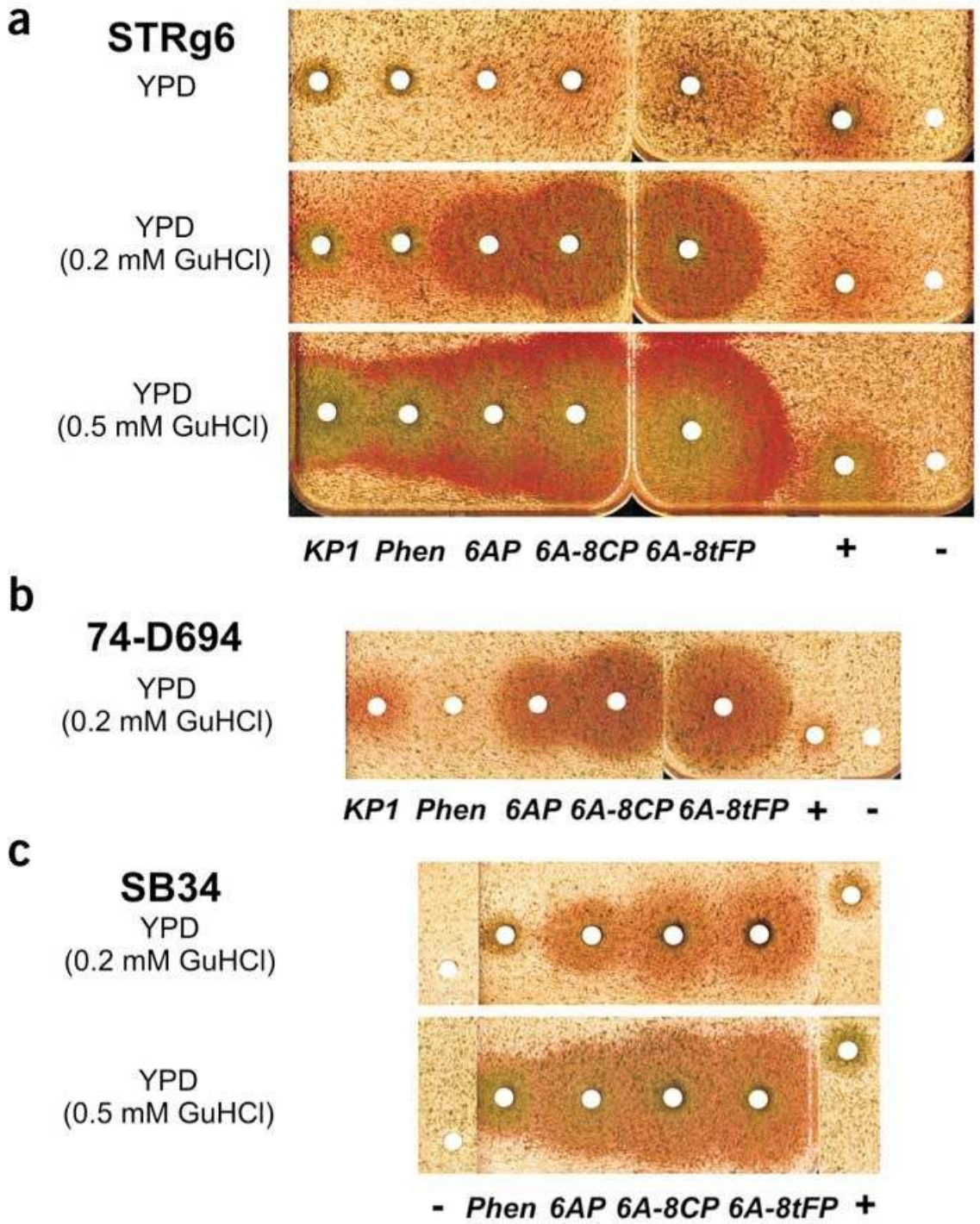


Figure 4 Bach et al.,

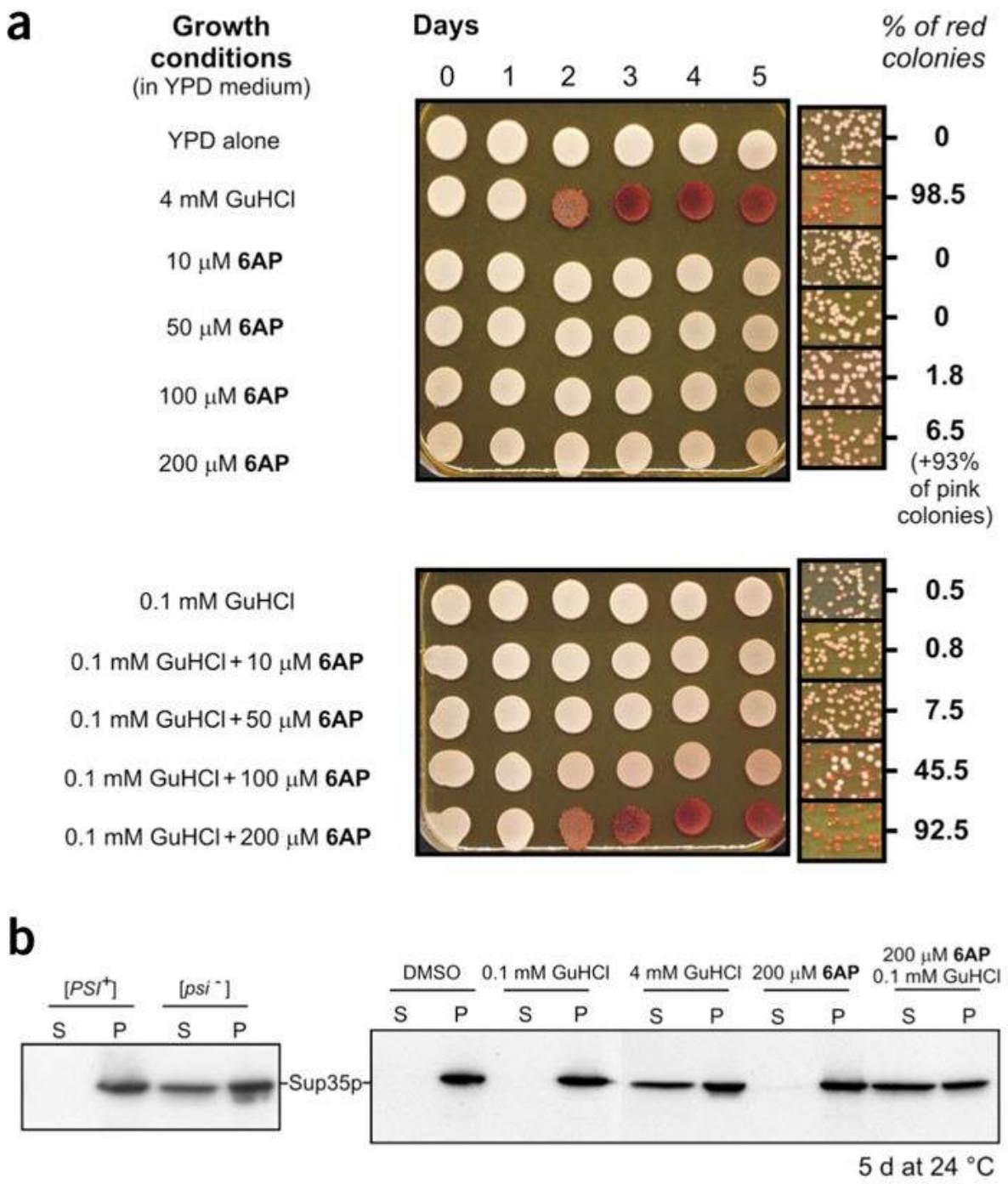
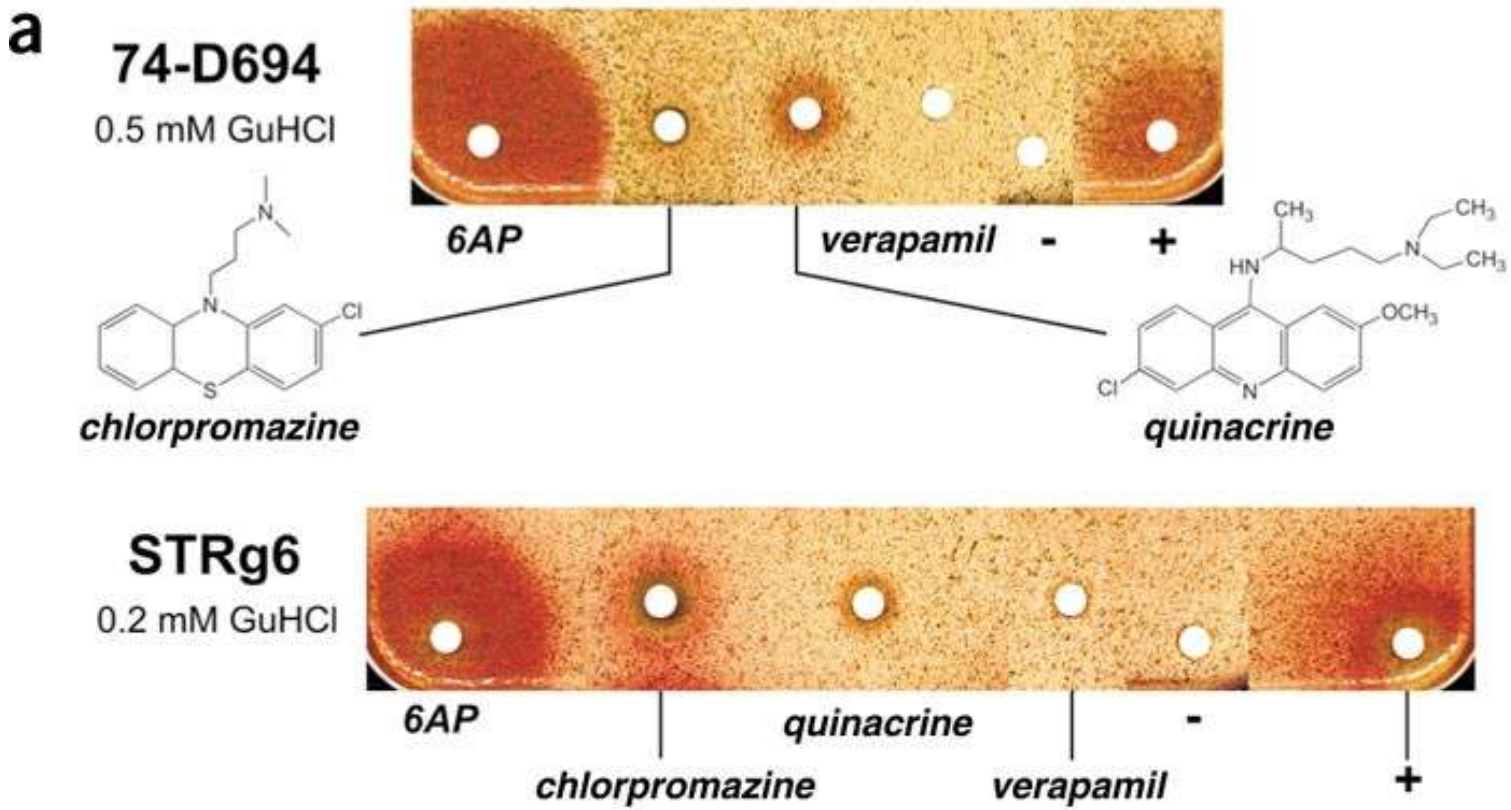


Figure 5 Bach et al.,



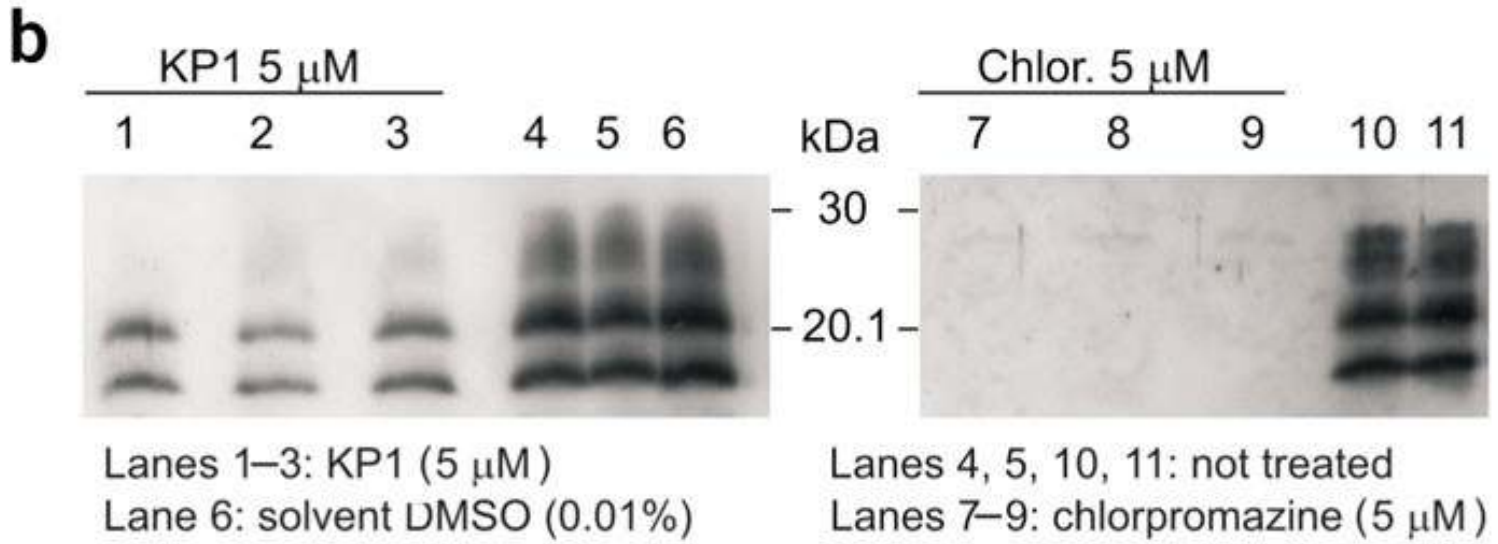


Table 1 Bach et al.,

Table 1 6AP effectively cures [*URE3*] prion in liquid culture

a USA reporter system[a]

Growth conditions (in YPD medium)	Number of colonies observed		Cure effectiveness
	USA medium	SD+Ura medium	
0.2 mM GuHCl	518	525	1.3%
5 mM GuHCl	30	1,132	97.3%
0.2 mM 6AP	87	510	82.9%
0.2 mM 6AP and 0.2 mM GuHCl	13	3,374	99.6%

b *ADE2* reporter system[b]

Growth conditions (in YPD medium)	Cure effectiveness observed after		
	7 generations	14 generations	20 generations
DMSO	0.7%	0.5%	0.5%
0.2 mM GuHCl	0.7%	1.6%	4.2%
5 mM GuHCl	76.6%	99.8%	99.9%
0.2 mM 6AP	4.6%	20.0%	67.2%
0.2 mM 6AP and 0.2 mM GuHCl	20.6%	35.0%	91.3%

[a][URE3] cells were kept in exponential growth phase in YPD liquid medium in the presence of the indicated concentration of the different molecules for 3 d (about 25 generations) at which time a fraction of the cell culture was removed and split in two halves: one was streaked on SD+Ura medium (where both [URE3] and [ure3-0] cells are able to grow) and the other on USA medium (where only [URE3] cells can grow). Note that 6AP substantially cured [URE3] cells and that this effect was enhanced in the presence of a sub-effective dose (200 μM) of GuHCl. [b]*ERG6wt* [URE3] cells (NT35) were maintained in exponential growth phase at 24 °C for 3 d (~20 generations). The percentage of red colonies (an indication of [URE3] curing efficiency) obtained by streaking an aliquot fraction of each cell culture at the end of the experiment was determined. At least 1,000 colonies were counted for each condition. Note that 6AP alone was able to substantially cure [URE3].