Published in final edited form as:

J Chromatogr A. 2015 January 30; 1379: 1–8. doi:10.1016/j.chroma.2014.12.014.

Prion protein-coated magnetic beads: Synthesis, characterization and development of a new ligands screening method[☆]

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Abstract

Prion diseases are characterized by protein aggregation and neurodegeneration. Conversion of the native prion protein (PrP^C) into the abnormal scrapie PrP isoform (PrP^{Sc}), which undergoes aggregation and can eventually form amyloid fibrils, is a critical step leading to the characteristic path morphological hallmark of these diseases. However, the mechanism of conversion remains unclear. It is known that ligands can act as cofactors or inhibitors in the conversion mechanism of PrP^C into PrP^{Sc}. Within this context, herein, we describe the immobilization of PrP^C onto the surface of magnetic beads and the morphological characterization of PrP^C-coated beads by fluorescence confocal microscopy. PrP^C-coated magnetic beads were used to identify ligands from a mixture of compounds, which were monitored by UHPLC–ESI-MS/MS. This affinity-based method allowed the isolation of the anti-prion compound quinacrine, an inhibitor of PrP aggregation. The results indicate that this approach can be applied to not only “fish” for anti-prion compounds from complex matrixes, but also to screening for and identify possible cellular cofactors involved in the deflagration of prion diseases.

[☆]Presented at the 41st International Symposium on High Performance Liquid Phase Separations – HPLC 2014, 10–15 May 2014, New Orleans, LA, USA.

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Keywords

Prion protein; Ligand fishing; Magnetic beads; Anti-prion compounds; Quinacrine; Screening method

1. Introduction

Prion diseases, classified as transmissible spongiform encephalopathies (TSEs), are characterized by protein aggregation and neurodegeneration and are invariably fatal due to the lack of effective treatment or cure. These diseases affect humans (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru and fatal familial insomnia) as well as other animals, including sheep (scrapie), deer and elk (chronic wasting disease), and cattle (bovine spongiform encephalopathy) [1–3].

Conversion of the native prion protein (PrP^C) into the abnormal scrapie PrP isoform (PrP^{Sc}), which undergoes aggregation, is the critical step leading to the development of these diseases [2,4]. The isoform normally found in the cell, PrP^C, is a soluble protein rich in α -helices, and the conversion of PrP^C into PrP^{Sc} involves refolding into a structure rich in β -sheets. PrP^{Sc} is partially resistant to digestion by proteases and is insoluble in aqueous solutions [5]. Due to its insolubility, PrP^{Sc} is susceptible to accumulation, forming fibers, that tend to aggregate in the central nervous system, resulting in the degeneration of brain function [6].

Human prion diseases can occur as sporadic, genetic or infectious disorders [7]. The sporadic form of prion disease is the most common, in which no mutations have been identified in the gene that encodes PrP^C, and the two isoforms (PrP^C and PrP^{Sc}) share the same amino acid sequence [8]. The conversion mechanism of PrP^C to PrP^{Sc} is still poorly understood, and several groups have suggested that an additional unknown cofactor could initiate or modulate this conversion [8–12]. Nucleic acids bind to PrP^C and, depending on their concentration and sequence, can exert catalytic or inhibitory effects on the conversion of PrP isoforms [9].

The search for a chemotherapeutic approach has focused on screening for anti-prion compounds to block the conversion of PrP^C to PrP^{Sc} [13]. These compounds may bind directly to PrP^C and stabilize its conformation, preventing the conversion to PrP^{Sc} and inhibiting aggregation.

Several compounds and synthetic peptides have been screened as potential antiprion agents, including polyanions (e.g. sulfated glycans) [11,14,15], curcumin [16], Congo red [17], cyclic tetrapyrroles [18], and others [13]. Antimalarial compounds were also investigated as potential antiprion agents; among them, quinacrine was identified as one of the most potent compounds, with reported IC₅₀ values ranging from 0.3 to 0.5 μ M in neuroblastoma cells [19,20]. Quinacrine has a high affinity for a PrP carboxyl-terminal binding site (Tyr225, Tyr226 and Gln227), with a K_D ranging from 1 to 5 mM [1,21,22].

Currently, the screening process for anti-prion compounds is often conducted through in vitro assays, using cell culture models, prevention of PrP amyloid formation and competitive binding assays. However, these approaches involve evaluating single isolated compounds and cannot isolate an active compound from a complex mixture.

Assuming that the prerequisite for anti-prion activity of a compound is affinity for the prion protein [23], as has been demonstrated with quinacrine, an affinity-based assay to isolate and identify anti-prion agents in compound mixtures would be beneficial. An approach that is gaining momentum for the identification of novel ligands from complex matrix is ligand fishing [24]. In this approach, the targeted protein is immobilized onto the surface of a stationary phase, predominantly, magnetic beads, and the resulting protein-based stationary phase is used to “fish” out the active compounds from a complex mixture [24,25].

Ligand fishing by derivatized magnetic particles was selected due to its versatility in the isolation of ligands from complex matrixes, such as natural product [26–30] and cell extracts [31,32], based on ligands/immobilized protein affinity. In this approach, the protein-coated magnetic beads are immersed into the complex matrix, and the non binders remain in solution or are removed by washing, while the bound ligands are eluted in an appropriate organic phase and can be identify by UHPLC–MS/MS.

To this end, we immobilized PrP^C onto the surface of magnetic beads, and the resulting protein coated beads were used for ligand fishing in a compound mixture. PrP-coated magnetic beads were characterized through fluorescence confocal microscopy. The proposed ligand fishing approach was validated by monitoring the capacity of the assay to isolate ligands from a mixture of compounds. Non-bound compounds and isolated ligands were quantified via UHPLC–ESI-MS/MS. The compound isolated from the mixture was the only one that demonstrated high inhibitory activity in aggregation formation studies, showing the correspondence between PrP^C affinity and anti-aggregation activity. Therefore, the results indicate that this approach is promising for the isolation of potential anti-prion compounds from complex matrixes, as well as of possible cofactors from cellular extracts, that could be involved in the deflagration of prion diseases.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were analytical or reagent grade and were used without further purification. Caffeine, quinacrine dihydrochloride and thiamine hydrochloride were purchased from Sigma Chemical (St. Louis, USA). Mouse recombinant prion protein (rPrP) 23–231 was expressed in *Escherichia coli* and further purified by high-affinity column refolding, as previously described [33]. Microtubes with a 2 mL capacity were obtained from Axygen Scientific (Union City, USA). The 1 µm BcMag[®] amine-terminated magnetic beads (50 mg/mL) were purchased from Bioclone Inc. (San Diego, CA). Ammonium acetate (99%), water and acetonitrile for mass spectrometry were obtained from Fluka (St. Louis, USA). Before being used in LC–MS/MS analysis, the buffer solutions were filtered through cellulose nitrate membranes (0.45 µm) provided by Phenomenex.

2.2. Apparatus

The chromatographic experiments were performed using a Shimadzu UHPLC system (Shimadzu, Kyoto, Japan), consisting of two LC 30AD pumps, an autosampler equipped with a 100 μ L loop (SIL 30AC) and a UV-visible detector (SPD-M30A) interfaced to an amaZon SL ion trap mass spectrometer with electro-spray ionization as the ion source (Bruker Daltonics). The MS parameters were set at 5 L min⁻¹ for the drying gas flow, 15 psi for the nebulizer pressure and 300 °C for the drying gas temperature. Data acquisition was accomplished on a Shimadzu CBM-20 A system interfaced with a computer equipped with Highstar 3.2 software (Shimadzu, Kyoto, Japan).

Far-UV Circular Dichroism – CD spectra were recorded in a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25 °C with circular 0.10-mm-pathlength cells. Buffer spectra were subtracted from each sample spectrum, and traces were collected with four accumulations each.

Light scattering (LS) measurements were recorded on an ISSPC1 fluorometer (ISS, Champaign, IL). LS at 90° was measured illuminating the samples at 320 nm and collecting LS from 300 to 340 nm.

Spectrophotometric assays were conducted in a Shimadzu UVmini-1240 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Prion protein immobilization onto the surface of magnetic beads

Prion protein was covalently immobilized onto the surface of silica-based magnetic beads (BcMag amine-terminated magnetic beads, Bioclone), following the protocol provided by Bioclone Inc., with slight modifications. First, 9 mg of BcMag amine-terminated magnetic beads (MBs) were washed with 1 mL of 10 mM pyridine buffer pH 6.0 in a 2 mL microtube. The supernatant was discarded after magnetic separation. The MBs were suspended in 1 mL of 5% glutaraldehyde and shaken for 3 h. After magnetic separation, the MBs were washed three times with 1 mL of 10 mM pyridine buffer at pH 6.0 to remove the unreacted glutaraldehyde. The amount of PrP used in the immobilization step was optimized and the following amounts were evaluated: 0.5; 1.0; 1.5 and 2.0 mg of PrP were incubated overnight with 3 mg of MBs in 10 mM pyridine buffer at pH 6.0 under gentle rotation at 4 °C. The highest yield was obtained using 1 mg of PrP^C to 3 mg of MBs. The stability of PrP^C under these conditions was ensured by the absence of aggregate formation and isoform conversion, tested by light scattering and circular dichroism measurements. Magnetic separation was then performed and the supernatant was used to estimate the amount of immobilized PrP, based on the molar extinction coefficient of PrP (63,495 cm⁻¹ M⁻¹ at 280 nM [34]). The PrP-coated MBs were incubated with 1 M glycine or 1 M hydroxylamine solution for 30 min at 4 °C to quench all residual aldehyde groups. The PrP-coated MBs were washed twice with 1 mL of 5 mM ammonium acetate buffer pH 7.4 and then stored in the same buffer at 4 °C.

Control-MBs (blank experiments) were prepared through the same procedure but without the addition of PrP.

2.4. Morphological characterization

PrP was labeled with amino-reactive fluorescein isothiocyanate (FITC) prior to immobilization to evaluate the success of the procedure by fluorescence confocal microscopy of the PrP^C-coated beads. PrP^C was incubated with FITC (Molecular Probes) at a molar ratio of 1:10 in 1 M phosphate buffer at pH 7.5 for 1 h at 4 °C. The unbound dye was removed by centrifugation using an Amicon Ultra filter unit with a molecular weight cut-off of 10 kDa (Millipore). Labeled PrP^C was suspended in 10 mM pyridine buffer pH 6.0 and passed through a syringe-driven filter unit with 0.22 µm pore size. The concentration of labeled protein and the efficiency of labeling were determined based on the molar extinction coefficient of rPrP^C 23–231 (63,495 cm⁻¹ M⁻¹ at 280 nm) and FITC (68,000 cm⁻¹ M⁻¹ at 280 nm). The labeled PrP^C was then immediately used in the immobilization procedure. The labeling ratio (*F/P*) was calculated using Eq. (1):

$$\text{Labeling ratio} = \frac{A_{492} \times E^{0.1\%} \times \text{MW}_{\text{PTN}}}{68,000 \times [A_{280} - (0.26 \times A_{492})]} \quad (1)$$

where A_{492} is the absorbance of PrP conjugated to FITC (FITC-PrP) at 492 nm, $E^{0.1\%}$ is the molar extinction coefficient of rPrP^C 23–231 at 280 nm, MW_{PTN} is the rPrP^C 23–231 molar weight (23,014 g mol⁻¹), and A_{280} is the absorbance of FITC-PrP at 280 nm.

MBs coated with FITC-PrP were visualized on an LSM 510 META laser-scanning confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with excitation by an argon ion laser at 488 nm and emission collected from 500 to 550 nm.

2.5. Library selection

To validate that immobilized PrP^C recognizes ligands that inhibit its aggregation, three compounds were selected: caffeine (no affinity for Syrian hamster prion protein) [35], thiamine (affinity for Syrian hamster prion protein) [35], and quinacrine (affinity for human and mouse prion proteins, inhibits PrP^C aggregation) [21,22]. The structures of these compounds are shown in Fig. 1.

2.6. UHPLC–MS/MS method

The experiments were conducted with an octadecyl column (Shim-pack HR-ODS, Shimadzu, 3 µm, 15 cm × 0.21 cm i.d.) using 0.1% formic acid in water:acetonitrile (80:20, v/v) as the mobile phase at a flow rate of 0.4 mL/min. The mass spectrometer was operated under positive ionization, with a drying gas flow of 5 L min⁻¹ and a temperature of 300 °C, in the MRM mode; thiamine (265.0 → 144.1; 122.1) was detected from 0 to 1.3 min, quinacrine (400.2 → 327.1; 244.0; 142.2) from 1.3 to 2.0 min, and caffeine (195.0 → 138.0; 110.0) from 2.0 to 3.0 min.

The thiamine, quinacrine and caffeine calibration curves were obtained using appropriate standard solutions containing the three compounds in 5 mM ammonium acetate pH 7.4. Sample solutions were prepared in triplicate at the following concentrations: 25, 50, 100, 200, 400 and 600 nM for thiamine and quinacrine and 50, 100, 200, 400 and 600 nM for

caffeine, in 5 mM ammonium acetate buffer at pH 7.4. Each calibration level contains the 3 analytes, except for 25 nM, which contains only thiamine and quinacrine, since the limit of quantification for caffeine is 50 nM. Then, 50 μ L samples were injected into the UHPLC–ESI-MS/MS system, and the calibration curves were constructed by plotting the peak area against the injected analyte concentrations.

The intra- and inter-day precision and accuracy of the method were evaluated by analyzing quality control samples at three different concentrations, namely 30, 250 and 600 nM for thiamine and quinacrine and 60, 250 and 500 nM for caffeine, in 5 mM ammonium acetate buffer at pH 7.4 for all samples. To this end, five samples of each concentration were prepared and analyzed on three non-consecutive days. The acceptance criterion for the limit of quantification was that the precision of three samples be under 20% variability, and the limit of detection was calculated taking a signal-to-noise ratio of 3. The selectivity of the method was assessed by blank (ammonium acetate buffer) injection.

2.7. Ligands fishing

Experimental conditions for the ligand fishing assay were optimized by determining the optimal incubation time and elution conditions. In the former case, 3 incubation times were tested, including 1 min, 2 min and 3 min. It was determined that 1 min was sufficient to bind quinacrine. The elution conditions were also optimized and the following elution buffers and incubation times were tested: 5 mM ammonium acetate pH 7.4: acetonitrile (90:10; 85:15; 80:20 and 70:30, v/v) during 1 min, 2 min and 3 min. It was determined that incubating the magnetic beads in 5 mM ammonium acetate pH 7.4: acetonitrile (85:15, v/v) elution buffer for 2 min, resulted in the greatest removal of bound quinacrine from the PrP^C coated magnetic beads. The optimal protocol is described as follows.

In a 2 mL microtube, 9 mg of PrP-coated MBs were suspended in 500 μ L of a mixture containing 300 nM of each analyte: thiamine, quinacrine and caffeine in 5 mM ammonium acetate buffer at pH 7.4. The tube was vortex-mixed for 1 min, and the supernatant was removed (S0, unbounded compounds). The PrP-coated MBs were washed twice for 1 min with 1 mL of 5 mM ammonium acetate buffer at pH 7.4 (S1 and S2). The PrP-coated MBs were subsequently vortex-mixed with 500 μ L of 5 mM ammonium acetate pH 7.4: acetonitrile (85:15, v/v) for 2 min to remove bound compounds (S3). The quantification of thiamine, quinacrine and caffeine in the samples S0, S1, S2 and S3 was performed using the UHPLC–MS/MS method. Each individual data presented is the average of triplicate bioaffinity assay and duplicate quantification measurements.

The same assay was performed using glycine-coated MB as “control study”, to verify the specificity of the observed interactions.

2.8. PrP^C aggregation assay

PrP^C aggregation was conducted by incubating 30 μ M of PrP^C and 300 μ M of caffeine, quinacrine or thiamine in 50 mM phosphate buffer with 100 mM NaCl at pH 7.4 under shaking at 300 rpm for 10 min at 65 °C. Aggregation controls without the addition of any compound were performed under the same conditions. PrP^C aggregation was then assessed by measuring the intensity of light scattered at 90° on an ISSPC1 fluorometer (ISS,

Champaign, IL). LS at 90° was measured by illuminating the samples at 320 nm and collecting LS from 300 to 340 nm. The PrP^C aggregation assays were performed in triplicate and the LS measurements in duplicate.

3. Results and discussion

The identification of compounds that can stabilize the PrP^C iso-form is an important task in the search for new drugs against prion diseases. A compound identified in a screening that binds to PrP^C may prevent its conversion to PrP^{Sc}, or may prevent PrP^{Sc} from interacting with PrP^C. In addition, the screening of potential cofactors that can catalyze the conversion of PrP^C to PrP^{Sc} within the cell is a promising approach to elucidate possible mechanisms for this conversion. Therefore, the development of analytical tools for the screening of PrP ligands remains important not only for understanding the development of these diseases but also for the identification of anti-prion compounds.

Herein, we have developed a bioaffinity chromatography method that can isolate a ligand with affinity for PrP^C from a mixture of compounds. In addition, it was demonstrated that the isolated ligand was active against the conversion of PrP^C to PrP^{Sc}.

3.1. PrP-coated magnetic beads characterization

PrP was covalently immobilized onto the surface of magnetic beads (MBs) via Schiff base formation between the aldehyde groups in the derivatized MBs and the amino groups of the lysine residues on the protein surface. It was previously demonstrated that the immobilization of proteins provides in some cases increased stability [36–38], due to the more restricted conformational mobility. A similar increase in stability was observed with the immobilization of PrP^C, preventing the conversion of PrP^C to PrP^{Sc} and PrP^{Sc}-like isoforms.

To characterize the PrP^C-coated MBs, PrP^C was labeled with FITC prior to the immobilization procedure. As the lysine residues on the protein surface are essential for subsequent immobilization, the FITC labeling reaction was conducted at pH 7.5, directing the conjugation to the amino terminal groups. The FITC:PrP^C labeling ratio (*F/P*) was calculated to be 0.568, indicating that PrP was nearly 56.8% labeled.

The labeled-PrP^C-coated magnetic beads were characterized by fluorescence confocal microscopy. The images were compared with images for non-labeled-PrP^C-coated magnetic beads and non-modified magnetic beads. Characteristic fluorescence was only observed in the labeled-PrP^C-coated MBs, while the MBs and the non-labeled-PrP^C-coated MBs did not fluoresce (Fig. 2). The images indicate both the success of the immobilization procedure and the uniformity of the MB coating.

3.2. Optimization of the immobilization procedure

The protocol for protein immobilization onto the surface of MBs requires the use of 10 mM pyridine buffer at pH 6.0, therefore, to ensure that PrP^C was immobilized in its native conformation, without the formation of aggregates, the stability of PrP^C in pyridine buffer was studied by light scattering and circular dichroism measurements. The protein was stable

in the immobilization procedure conditions (overnight in 10 mM pyridine buffer at pH 6.0 and 4 °C, under gentle rotation). To optimize the efficiency of the PrP^C immobilization procedure, several amounts (0.5; 1.0; 1.5 and 2.0 mg) of PrP were incubated with 3 mg of MBs, and the amount of PrP^C bound was estimated based on the molar extinction coefficient of PrP^C before and after incubation with the MBs. The best ratio of PrP^C:MBs evaluated was 1:3 (mg), with an average of 0.127 ± 0.042 mg of immobilized PrP^C per mg of MBs (average of 5 immobilization procedures, with triplicate quantification measurements), resulting in 38.1% of immobilization yield.

The PrP^C-coated MBs were endcapped with glycine and hydroxylamine, and the specificity of the interactions between PrP^C-coated MBs and the selected compounds was later investigated.

3.3. UHPLC–MS/MS method validation

Calibration curves to quantify thiamine, quinacrine and caffeine were constructed using the reference compounds. A typical chromatogram is presented in Fig. 3A and the MS/MS spectra for thiamine, quinacrine and caffeine are shown in Fig. 3B, C and D, respectively. Curves for simultaneous quantification of the selected compounds were linear in the range studied for each compound, with mean correlation coefficient values ($n = 3$) of 0.99 or higher, accuracy and RSD values for QC samples in the range of 88.3–112.9% and 0.391–12.1%, respectively.

Based on a signal-to-noise ratio of 3:1, the limit of detection values attained for thiamine, quinacrine and caffeine were 5.0, 5.0, and 15 nM, and the limit of quantification values were 25, 25 and 50 nM, respectively. The developed UHPLC–MS/MS method was employed to quantify these compounds in the ligands fishing assay.

3.4. Ligands fishing assay

To investigate whether the PrP^C-coated MBs can be used to discriminate inhibitors from non-inhibitors of PrP^C aggregation based on their affinities for the target, PrP^C beads were incubated with an equimolar (300 nM) solution containing thiamine, quinacrine and caffeine. Fig. 4 illustrates the workflow for the PrP^C ligands fishing assay.

Initially, magnetic beads were prepared in two batches: one endcapped with glycine, as recommended by Bioclone Inc., and a second endcapped with hydroxylamine [32]. The results obtained using PrP^C-coated MBs endcapped with glycine and hydroxylamine were compared with the results obtained using MBs coated with glycine and hydroxylamine, without PrP^C, to verify the binding specificity. PrP^C-coated MBs endcapped with glycine captured $32.9 \pm 8.4\%$ of the quinacrine from the mixture (Fig. 5), while the MBs coated with glycine did not retain any tested compound. Non-specific binding was observed when MBs were endcapped with hydroxylamine (data not shown); therefore, glycine-endcapped MBs were selected for subsequent experiments.

The reusability of PrP^C-coated MBs in the fishing assays was investigated using the same batch of MBs in successive ligands fishing experiments with caffeine, quinacrine and thiamine. During two successive experiments, the capacity for quinacrine retention was

reduced by only $3.2 \pm 0.8\%$, showing that the PrP^C-coated MBs can be reused in several assays.

3.5. Effect of the selected compounds on PrP aggregation

The conversion of PrP^C to the infectious PrP^{Sc} isoform leads to the refolding of the alpha helical- and coil-rich structure into a beta sheet-rich structure. The PrP^{Sc} isoform tends to aggregate due to its insolubility; protease-resistant aggregates then accumulate in the brain, which is a feature of TSEs. Therefore, there is vast interest in the identification of a compound that can stabilize PrP^C and prevent its conversion to PrP^{Sc}.

The assay described herein is presented as an initial screening method to rapidly isolate ligands with affinity for PrP^C as potential aggregation inhibitors. Therefore, to verify the correspondence between affinity and anti-aggregation activity, the selected compounds were evaluated for their capacity to prevent PrP^C aggregation.

Several methods can be employed to assess PrP^C aggregation, such as temperature [39], agitation [40], incubation with nucleic acids [4] and lipids [41,42]. From these, the thermal aggregation protocol was selected to evaluate the aggregation inhibition of the studied compounds. PrP samples at 30 μ M were incubated with caffeine, quinacrine or thiamine at 300 μ M and aggregation assays were conducted at 65 °C and 300 rpm for 10 min, based on previously reported studies [43]. As positive and negative controls we used a sample containing only PrP^C at 30 μ M submitted to the same thermal treatment, and PrP at 30 μ M not submitted to the thermal treatment, respectively. Aggregation was subsequently monitored by increased light scattering at 300–340 nm, as illustrated in Fig. 6A and B.

This study demonstrated a clear correspondence between affinity and anti-aggregation activity for the studied compounds. Aggregates scattered strongly due to their higher mass weights. The results indicated aggregate formation in the positive control experiment, as well as the samples containing caffeine or thiamine while quinacrine, the only retained compound by the fishing experiment, demonstrated significant inhibition of PrP^C aggregation (100% at 300 μ M), with a similar LS value to the negative, non-aggregated, control.

4. Conclusions

The results reported herein describe a rapid, specific and reliable method to screen for new potential anti-prion agents. PrP^C can be immobilized onto the surface of magnetic beads and applied in affinity studies to isolate potential anti-prion agents from compounds mixtures. The PrP^C-coated MBs retained their capacity to retain active ligands and the beads can be applied to successive experiments.

In the search for PrP ligands, fishing assays are a useful tool due to their ability to isolate potential unknown ligands from complex matrixes. Therefore, this method can be applied not only as a first trial of potential inhibitors of PrP^C aggregation, but also in the screening and identification of possible cellular cofactors involved in the deflagration of prion diseases.

Acknowledgments

This work was funded by grants from the National Council for Scientific and Technological Development (CNPq) and the Carlos Chagas Filho Foundation for Research Support in the State of Rio de Janeiro (FAPERJ). We also thank Eduardo R. dos Santos and Prof. Dr. Ronaldo da Silva Mohana Borges from CEMBIO-CCS-UFRJ for the UHPLC-MS/MS availability. This work was also supported in part by Intramural Research Program at National Institute on Aging (RM).

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Fig. 1.
Structures of the selected compounds.

Fig. 2.

Images captured by fluorescence confocal microscopy for examination of the MBs, non-labeled-PrP^C-coated MBs and FITC-labeled-PrP^C-coated MBs in the FITC fluorescence channel, bright-field and merged images.

Fig. 3. UHPLC–MS/MS chromatogram (A) for the separation of thiamine (0.83 min), quinacrine (1.5 min) and caffeine (2.2 min) at 600 nM in 5 mM ammonium acetate pH 7.4. MS/MS spectra for thiamine (B), quinacrine (C) and caffeine (D) at 600 nM.

Fig. 4.
Steps involved in the PrP^C ligands fishing assay.

Fig. 5. Ligands fishing by PrPC-coated magnetic beads. (A) Assay with glycine-coated MBs, (B) assay with PrPC-coated MBs endcapped with glycine. MBs were immersed in a mixture containing 300 nM of each selected compound. The S0 fraction represents non-bound compounds; S1 and S2 were obtained by subsequent washing with 5 mM ammonium acetate buffer at pH 7.4; and the S3 fraction was collected by elution of the retained compounds with 5 mM ammonium acetate pH 7.4: acetonitrile (85:15, v/v). The fractions were analyzed by the developed and validated UHPLC–MS/MS method. Error bars correspond to the standard deviation of the triplicate bioaffinity assay and duplicate quantification measurements.

Fig. 6.

(A) Light scattering values obtained for samples containing 30 μM PrP^C after shaking at 65 °C and 300 rpm for 10 min. Excitation was at 320 nm and emission was read at 300–340 nm. (B) Representative plot correspondent to a replicate of the LS measurements of the samples containing only PrP^C (positive control), and PrP^C incubated with thiamine, quinacrine and caffeine at 300 μM after thermal treatment at 65 °C and 300 rpm. Negative control corresponds to LS measurements of PrP^C samples before thermal treatment. Error bars correspond to the standard deviation of the triplicate aggregation assay and duplicate quantification measurements.