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Screening a library of potential prion therapeutics against cellular prion proteins and insights into their mode of biological activities by surface plasmon resonance

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Abstract

The conversion of cellular prion protein (PrP^C) to the protease resistant isoform (PrP^{Sc}) is considered essential for the progression of transmissible spongiform encephalopathies (TSEs). A potential therapeutic strategy for preventing the accumulation of PrP^{Sc} is to stabilize PrP^C through the direct binding of a small molecule to make conversion less energetically favourable. Using surface plasmon resonance (SPR)-based technology we have developed a procedure, based on direct binding, for the screening of small molecules against PrP^C immobilized on a sensor chip. In this paper we report some problems associated with the immobilization of PrP^C onto the sensor surface for conducting drug screening and how these problems were overcome. We demonstrated that the conformational change of PrP^C on the chip surface leads to increased exposure of the C-terminal which was observed by the increase in quinacrine binding over time, and loss of heparin binding to the N-terminal. In addition, we also report the results of the successful screening of a library of 47 compounds of known activity in cell line or cell free conversion studies for direct binding to three forms of PrP^C (huPrP^C, t-huPrP^C and moPrP^C). These results show the usefulness of this technique for the identification of PrP^C binding ligands and to gain some insight as to their potential mode of action.

Keywords: TSE; CJD; Prion protein; SPR; Library screening; Carboxymethylated dextran; Immobilization; Conformational change

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases affecting animals and humans [1]. The human form of TSE is known as Creuzfeld–Jakob disease (CJD). Formation of amyloidal deposits in affected brain is a hallmark of the disease similar to many other neurodegenerative conditions such as Alzheimer's disease. These deposits are constituted mainly of aggregated prion proteins in a misfolded state. The cause for the misfolding/aggregation of the host proteins at a molecular level is unknown and occurs spontaneously (sporadic type) or can be attributed to genetic reasons (familial type). Strikingly different from other neurodegenerative diseases, TSEs are also infective (iatrogenic type) and can be transmitted via transplants, contaminated biological products from cadavers, blood transfusion, contaminated surgical instruments and ingestion of infected materials. The latter has been observed in humans after consumption of cattle affected by bovine spongiform encephalopathy (BSE) and has been termed variant CJD (vCJD). No effective treatments for CJDs are currently available, nor has a bio-molecular component been fully validated as a drug target. However, it is believed that the formation of protease resistant insoluble prion protein (PrP^{Sc}), which is the main component of amyloidal deposits, from the cellular prion protein (PrP^C), is essential for the progression of the disease. Therefore, both PrP^{Sc} and PrP^C are currently being used as potential drug targets [2].

PrP^C is a membrane protein of unknown physiological function [3–5]. It consists of an unstructured, flexible N-terminal domain (AA23–110) which contains five octarepeats

Abbreviations: TSE, transmissible spongiform encephalopathy; CJD, Creuzfeld–Jakob disease; PrP^{Sc}, protease resistant prion protein; PrP^C, cellular prion protein; GAG, glycosaminoglycan; SPR, surface plasmon resonance; RU, response units; huPrP^C, full length human PrP^C; t-huPrP^C, truncated human PrP^C; moPrP^C, full length mouse PrP^C

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(AA51–91), a globular C-terminal domain (AA111–230) which contains two glycosylation sites (Asn181, Asn197) [6] and a glycosylphosphatidyl-inositol (GPI) anchor (Ser230) [7]. Several compounds are known to interact directly with PrP^C including the natural binding partners heparin and glycosaminoglycans (GAGs) [8–11], copper [12–16], nucleic acids (RNA and DNA) [17–21], plasminogens [22,23], laminin receptors [24], PrP fragments and PrP^C itself [23]. Other binding partners have been reported including various antibodies [25,26], Congo Red and quinacrine [27]. However, only one compound, quinacrine, can be considered as a small drug-like molecule.

Several studies have reported binding events between PrP^C and antibodies, heparin and plasminogen using surface plasmon resonance (SPR) [8,23,25,28,29]. In all of these studies the quantity of PrP^C immobilized on the SPR sensor chip was low (500–1000 RU). Baseline instability was not reported in any of these studies although changes in binding behaviour of PrP^C to some antibodies raised against the N-terminal epitopes over time was observed [26].

As part of an ongoing medicinal chemistry program towards the automated screening of potential therapeutic compounds against CJD, a direct binding assay for small ligands with recombinant human prion protein ($huPrP^C$) was explored. SPR was selected as an assay platform due to the ease of assay development, medium sample throughput and automated sample handling, as well as the reported success of the technique as a binding assay for small drug-like molecules against other drug targets [30–32]. However, it is well known that prion protein binds strongly to metal surfaces [33–35]. It may also bind to the carboxymethylated dextran (CM-dextran) on the gold surface because, structurally, CM-dextran resembles heparin, which is known to bind to prion proteins [8–11]. For the convenience and optimal throughput in drug screening for prion lead discovery, immobilizing PrP^C on the surface and injecting a compound of interest to study their interactions would be ideal. But drug like compounds normally have a low molecular weight between 300 and 800 Da. To observe binding between a prion protein and those compounds it is essential to be able to immobilize prion proteins at a level between 3000 and 10 000 RU and keep the baseline as stable as possible. A number of problems were encountered and overcome during the development of an optimal protocol for the use of SPR for screening and mechanistic studies.

In this paper we describe the problems associated with the high $huPrP^C$ immobilization levels required for drug screening, possible causes and changes to the immobilization procedure that overcame these problems. We demonstrate that it is a repositioning of PrP^C on the sensor surface that leads to the increased exposure of the C-terminal, and an increase in quinacrine binding. Interactions between PrP^C and CM-dextran also resulted in the loss of heparin binding to the N-terminal. We also report the results of the successful screening of a library of 47 compounds of known activity in cell line or cell free conversion studies for direct binding to three forms of PrP^C ($huPrP^C$, $t\text{-}huPrP^C$ and $moPrP^C$).

2. Materials and methods

2.1. Materials

N-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3–diethylaminopropyl) carbodiimide hydrochloride (EDC), 1 M ethanolamine, HBS-EP buffer, surfactant P20, regeneration solution (10 mM glycine–HCl, pH 3.0) and CM-dextran (MW ≈ 13 000 Da) were purchased from BIAcore. Sodium phosphate, ethylenediaminetetraacetic acid (EDTA), sodium chloride, sodium hydroxide and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich. Bovine serum albumin (BSA) was purchased from New England BioLabs. Recombinant full length human prion protein ($huPrP^C$), truncated human prion protein ($t\text{-}huPrP^C$) and full length mouse prion protein ($moPrP^C$) were kindly provided by the Institute for Animal Health (Compton, UK).

All compounds in the test library were purchased from Sigma–Aldrich except the dicarbonitrile compounds, which were from Maybridge, UK and *Z*-phenyl arginyl-7-amido-4-methyl coumarin, which was from CN-Biosci, Japan. Compounds were used as supplied without purification.

2.2. Preparation of the SPR sensor surface

Biacore 3000 is a commercially available SPR biosensor instrument. It involves attaching one interacting partner to the surface of a sensor chip and passing the solution containing the other interaction partner(s) over the surface. The binding of molecules to the target attached to the sensor surface generates an evanescent response which is proportional to the bound mass. No sample labeling is required. In our experiments it was decided to immobilize prion protein on the surface instead of the compounds to be studied for convenience and throughput.

Experiments were performed using a BIAcore 3000 (BIAcore, Uppsala, Sweden) equipped with a CM5 sensor chip. HBS-EP buffer was used for immobilization of the protein. Prior to immobilization the folded state of the protein was assessed by acquiring circular dichroism (CD) spectra and comparison with the authentic sample as a quality control tool.

2.2.1. Standard immobilization procedure

CM-dextran on a CM5 sensor chip was activated by mixing equal volumes of 100 mM NHS and 400 mM EDC followed by injection of the mixture over the sensor chip surface for 7 min at a flow rate of 5 μL/min. The $huPrP^C$ to be immobilized was injected over the surface for 7 min. The unreacted sites on the sensor chip surface were blocked by injection of 1 M ethanolamine, pH 8.5 for 7 min.

2.2.2. Investigations into optimal immobilization conditions, baseline instability, and its causes

A series of investigations were carried out to assess whether the commercially available SPR system Biacore 3000 is a viable tool for screening drug like libraries in lead discovery for prion disease, and to develop and validate the assay in terms of the high immobilization level, detection limit and baseline stability

when PrP^C is required to be immobilized onto the sensor chip surface.

2.2.2.1. Selection of optimal concentration of prion protein for immobilization. To a given immobilization level of a protein, the theoretical maximum binding resonance units (RU_{max}) for a ligand can be calculated using the following formula:

$$RU_{max} = \left(\frac{\text{RU immobilised protein}}{\text{MW protein}}\right) \times \text{MW ligand}$$

To account for the detection limits of the machine and baseline noise (5–10 RU), 40–100 RU for a small molecular weight compound is generally required at a given immobilization level of the protein. For a compound like quinacrine, if a binding of 100 RU is required to be observed on the chip surface, the level of prion protein required to be immobilized is around 4000 RU, calculated by the above formula or vise versa.

To achieve this, a stock solution of $huPrP^C$ (0.72 mg/mL) was diluted in 10 mM acetate buffer at pH 5.5 to concentrations of 10, 5.5 and 2 μg/mL, respectively, and these concentrations were used for immobilization. An optimal protein concentration of 2 μg/mL was selected as the universal protein concentration for all immobilizations as this was sufficient to achieve an immobilization level of approximately 4000 RU. A set of known PrP^C binders including quinacrine, Congo Red and heparin at 40 μM were used to assess the sensitivity of the system at each immobilization level. Imipramine and caffeine was used as negative controls.

2.2.2.2. Causes for the baseline increase and methods to improve baseline stability. The binding of quinacrine and heparin were recorded over a 72 h period at an $huPrP^C$ immobilization level of 4000 RU. The sensor surface, prepared using the standard immobilization procedure, was also left running over a period of 14 h to assess the baseline stability. The scale of the baseline increase was recorded.

The following experiments were designed to investigate the causes of the baseline increase and how to eliminate them:

(a) 8 M urea and 6 M guanidine hydrochloride solutions were injected over the prepared surface to investigate whether the increased baseline was due to the dimerisation of prion protein on the surface.
(b) PrP^C was mixed with different molecular weight dextrans and CM-dextran prior to the injection over the sensor surface to investigate whether the binding of PrP^C to CM-dextran contributed to the loss of heparin binding to PrP^C.
(c) $HuPrP^C$ was injected over the sensor surface without activation of the surface to see if the protein attached to the surface via physical adsorption. Different washing buffers with various properties and concentrations were used to remove the adsorbed protein.
(d) The optimal buffer was used to remove the physically adsorbed PrP^C at time intervals between 5 and 30 min to select the optimal time for applying the washing buffer to obtain an optimal surface for binding studies and screening.

2.2.3. Optimized immobilization procedure

The $huPrP^C$ was immobilized in the same manner as described for the standard immobilization procedure. The prepared surface was washed thoroughly immediately after the ethanolamine blocking step, by three consecutive injections of 25 mM NaOH/1 M NaCl solution at an interval of 8 s. The surface was then equilibrated with the running buffer for 30 min prior to the injection of sample solutions.

2.3. Ligand binding and screening

2.3.1. Sample preparation

The compounds tested can be divided into two groups: water soluble and water insoluble. 800 μM stock solutions of all water soluble compounds were made using running buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20). Similarly, 800 μM stock solutions of all water insoluble compounds were made in 100% DMSO. Both stock solutions were diluted to the required 40 μM concentration using the same running buffer.

2.3.2. Screening procedure

All assays were run at 25 °C with a flow rate of 30 μL/min, using phosphate buffer as a running buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20). $HuPrP^C$ was immobilized in flow cell 2, $t\text{-}huPrP^C$ was immobilized in flow cell 3 and $moPrP^C$ was immobilized in flow cell 4, flow cell 1 was used as a reference. The experiments were designed to systematically compare compounds binding towards $huPrP^C$, $t\text{-}huPrP^C$ and $moPrP^C$. Compounds which bound to all three types of PrP^C are more likely to be C-terminal binders while those which bound to both full length protein but not to the truncated form should be N-terminal binders. Compounds may also show selectivity between human and mouse PrP^Cs. Eventually a correlation between binding to $moPrP^C$ and PrP^{Sc} inhibition in mouse cell lines, $huPrP^C$ binding and cell line activity could be explored. This interaction can provide an insight into the possible modes of action of the cell line active compounds. For water soluble compounds, each analytical cycle consisted of running buffer for 1 min (stabilization phase), a sample injection at 40 μM in running buffer for 1 min (association phase) and running buffer for 3 min (dissociation phase). Subsequent surface regeneration at a flow rate of 35 μL/min was carried out using two regeneration solutions: (i) a 30 s injection of 25 mM NaOH/1 M NaCl with 0.0005% SDS (pH 8.5), (ii) a 35 s injection of 10 mM glycine–HCl (pH 3.0). After regeneration the surface was allowed to stabilize for 1 min. The total run time was approximately 8 min/cycle. The sensor chip was usually discarded after 5 days. The data was anlaysed and the binding was expressed as $\%RU_{max}$ as defined by the following equation for a 1:1 stoichiometry:

$$\%RU_{max} = \left(\frac{\text{RU of compound}}{\text{theoretical RU of the compound}}\right) \times 100$$

For compounds using DMSO as a co-solvent, 6.5% DMSO was added to the running buffer and a DMSO calibration using buffer samples containing 5.5–7.5% DMSO was carried out at the

beginning and the end of each block of 10 compounds to correct for solvent effects.

2.4. Binding analysis

Binding was measured against both huPrP^C and moPrP^C for a selection of compounds. For aqueous soluble compounds the PrP^C surface was prepared using the optimal immobilization procedure. A series of concentrations were injected at a flow rate of 30 μL/min over immobilized PrP^C, and the reference surface at 25 °C. A single injection of each solution was carried out from the lowest to the highest concentration and sensorgrams were recorded for each injection. The sensor surface was regenerated with a pulse of 25 mM NaOH/1 M NaCl between each injection. For compounds requiring DMSO as a co-solvent, a series of concentrations were injected in triplicate, in an ascending order, each containing 6.5% DMSO. DMSO calibration was performed and the %RU$_{max}$ was calculated.

The sensorgrams were then overlaid and the data point obtained at the end of the association time was plotted against the concentration and analysed using Sigma plot to give dissociation constant (K_D) for each compound at equilibrium. For the data which could not be fitted using Sigma plot the K_D was estimated by interpolation of the concentration at 50%RU$_{max}$.

3. Results and discussion

3.1. Preparation of sensor surface

The standard immobilization conditions, as recommended by the manufacturer of the BIAcore 3000, were followed using huPrP^C to establish a general protocol. Various protein concentrations lead to immobilization levels from 500 to 10 000 RU. In order to accurately measure the interaction of small compounds with huPrP^C, an immobilization level of approximately 4000 RU was selected. A protein concentration of 2 μg/mL was used for the immobilization. A set of compounds including quinacrine, Congo Red and heparin at 40 μM each were injected to evaluate the binding assay (Table 1). Binding was observed for quinacrine, heparin and Congo Red, but not for imipramine and caffeine. However, a regeneration step for removing bound compounds was required for Congo Red and heparin, whereas quinacrine dissociated completely without the need for any regeneration. Different regeneration solutions were evaluated for their ability to regenerate the surface. It was found that Congo Red could only be removed using a sodium hydroxide-based regeneration solution (100 mM NaOH). Unfortunately at such a concentration there was a risk of causing damage to the immobilized protein, therefore a more dilute regeneration solution of 25 mM NaOH/1 M NaCl with 0.0005% SDS followed by 10 mM glycine–HCl, pH 3.0 was selected.

During these measurements an average increase of the baseline of up to 1.0 RU/min was observed, which was too high to achieve the reproducibility required for our assay. Interestingly, an increase in relative binding over a long time period (0, 24, 48 and 72 h) was also observed for quinacrine (Fig. 1A), which was initially attributed to a lack of baseline stability. During the evaluation of the regeneration solutions, no change of the baseline level other than the baseline increase was observed indicating that even at various pH levels and/or ionic concentrations the problem remained. Furthermore, the baseline increase could not have been caused by contamination of the needle or injector block with huPrP^C. A decrease in SPR response with time would be expected because a variation in the amount of huPrP^C being washed from the other surfaces onto the flow cells upon use of regenerating solutions would occur.

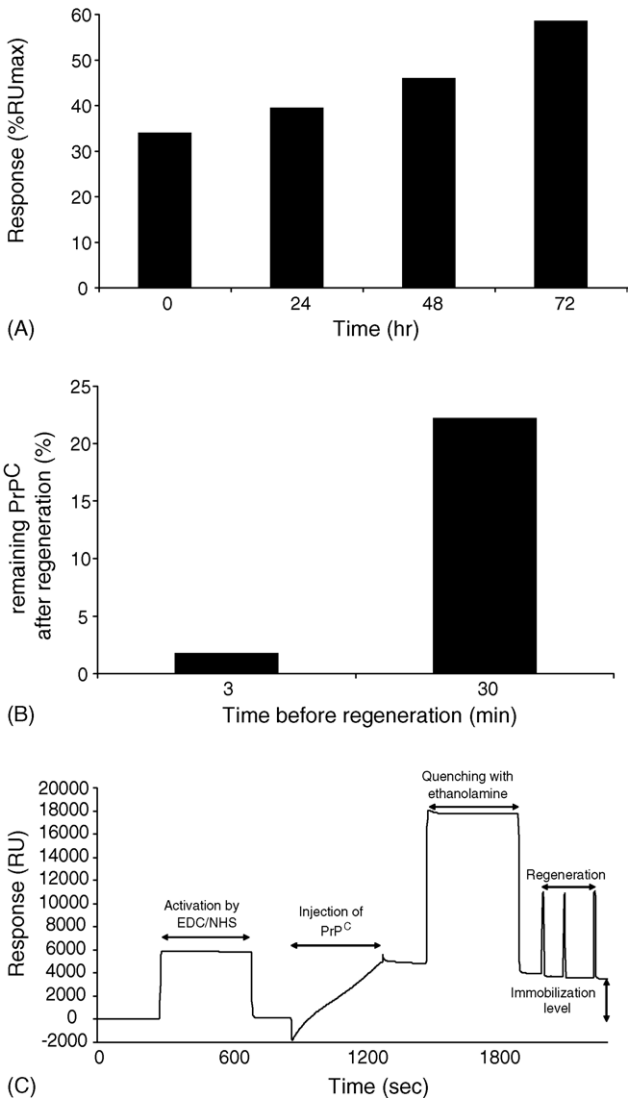


Fig. 1. (A) Bar chart of quinacrine binding to immobilized huPrP^C at 0, 24, 48 and 72 h after immobilization. (B) Time dependant removal of adsorbed huPrP^C from CM5 chip by 100 mM HCl regeneration solution (three 30 s pulses). (C) A typical sensorgram obtained during the immobilization of huPrP^C applying the modified immobilization protocol. A clear reduction of the baseline during the regeneration was observed.

To eliminate the possibility of dimerisation of huPrP^C on the chip surface or tight protein–protein interactions between huPrP^C on the surface, two denaturing solutions (8 M urea solution and 6 M guanidine hydrochloride solution) were injected (data not shown). Neither solutions led to a reduction in baseline level, hence it was assumed that the immobilized protein exists

Table 1
Ligand/huPrP^C binding measured by SPR

	Quinacrine	Heparin	Congo Red	Imipramine	Caffeine
Response after association (RU)	41.9 ± 1.8	101.8 ± 5.1	817.2 ± 105.5	−9.4 ± 1.0	−3.3 ± 0.4
Response after dissociation (RU)	−0.4 ± 0.4	85.7 ± 7.3	507.5 ± 100.3	0.1 ± 0.24	2.1 ± 0.3

Binding of five compounds at 40 μM (positive and negative controls) to huPrP^C after association and dissociation. Average of three consecutive injections with standard deviations.

in a monomeric state and protein–protein interactions were not the cause for the baseline drift.

The positive drift of the baseline, the increased binding of quinacrine and decreased binding of heparin over the time drove us to investigate the possible interactions between the protein and CM-dextran. HuPrP^C was injected onto a sensor surface (CM 5 chip) which was not activated. As expected, the protein was physically adsorbed onto the sensor surface due to the ionic interactions of the positively charged huPrP^C with the negatively charged sensor surface. When mixed with different molecular weight dextran a significant reduction in PrP^C absorption was observed while mixing with CM-dextran completely inhibited PrP^C absorption. This showed that PrP^C interacts with dextran and binding of PrP^C to the negatively charged CM-dextran led to PrP^C changing from positively charged to negatively charged, therefore unable to absorb onto the negatively charged surface via electrostatic interactions. Attempts to remove the protein by injection of either acidic solution (to protonate the surface) or a basic solution (to deprotonate the huPrP^C) were only partly successful, indicating that the observed interactions were not exclusively due to physical absorption caused by ionic interactions. Furthermore, removal of the adsorbed huPrP^C from the surface was time dependent. Up to 95% of the adsorbed huPrP^C was removed if the surface was washed with a 100 mM HCl solution within 5 min. A longer incubation time between the protein and the surface led to an increase of the protein, which could not be removed by the washing procedure (Fig. 1B). Similarly, when a 25 mM NaOH solution was injected 15 min after the adsorption of huPrP^C, 95% of the huPrP^C was also removed.

Two washing procedures were tested, one of which was regeneration before the ethanolamine blocking step while the other was regeneration after the blocking step. Both regenerations were equally efficient at removing huPrP^C, and reduced the baseline increase, but washing after the blocking step was selected for its ease of integration into the standard settings (Fig. 1C). A 25 mM NaOH/1 M NaCl regeneration solution was used for the procedure as this solution has been shown previously to be ideal for removing adsorbed huPrP^C. Using this modified immobilization procedure the baseline drift was reevaluated. Four different surfaces were produced on a CM5 chip; a blank surface (no protein), a BSA surface (as protein reference), a huPrP^C surface (prepared using the new immobilization procedure) and a huPrP^C surface (prepared using the standard immobilization procedure). The baseline was observed over 100 injections of running buffer, totaling approximately 2000 min. The blank surface showed a very small negative drift (−0.0170 ± 0.0045 RU/min) whereas the BSA surface showed a small positive drift (0.0150 ± 0.0023 RU/min) of similar magnitude. The huPrP^C surfaces showed a significantly bigger drift using the standard procedure (0.3338 ± 0.1414 RU/min). However, using the modified immobilization reduced the baseline drift by more than 50% when compared to the standard procedure (0.1346 ± 0.0869 RU/min).

The surfaces generated by the modified immobilization procedure were evaluated again against our set of test compounds. The binding of quinacrine remained, but still increased, whilst heparin binding decreased rapidly over time. Regeneration of the protein on the surface was time dependant indicating a certain repositioning of the huPrP^C on the chip. However, there was no direct evidence from our experiments to indicate a change in the tertiary structure of the huPrP^C on the chip surface, as was proposed by a study describing reduction in antibody binding towards the huPrP^C [26]. It is quite possible that immobilized PrP^C gradually binds to CM-dextran on the surface. This could cause the repositioning of the protein on the surface leading to greater exposure of the C-terminal and therefore an increase in quinacrine binding, while the N-terminal becomes occupied by CM-dextran, therefore the heparin binding is lost. Interestingly, the antibodies showing the most severe loss of binding to the prion protein in the study previously mentioned were raised against epitopes of the N-terminal region [26]. This loss of antibody binding could be explained by the interaction observed between the huPrP^C and the CM-dextran. Furthermore, the low level binding of heparin (∼3%RU$_{max}$) immediately after the immobilization indicates that the binding between PrP^C and CM-dextran is a very fast and irreversible process (reaction completes within 15 min), or that CM-dextran holds the PrP^C on the surface in a specific conformation which makes the binding site for heparin unavailable.

3.2. Screening of a library of cell line active and inactive compounds

The existing screening protocol for compounds as potential anti-prion agents focuses on the use of cell and animal models and cell free conversion assays. Compounds which have been screened in such systems were assessed for their ability to bind to PrP^C by the SPR method described above. Compounds which are known to be both active and inactive were screened. This included nucleic acid derivatives, amino acid and peptide derivatives, antibiotics, dyes and a variety of other compounds such as phenothiazine derivatives, all of which were soluble in either water or DMSO [2,36–44]. For many of the active compounds the mechanism of action is unknown, however, by assessing the ability of active and inactive compounds to bind to huPrP^C, as well as to t-huPrP^C and moPrP^C, it might be possible to begin to

understand these mechanisms. By comparing the results of this study with those of published work in which the compounds have been screened for their ability to inhibit conversion of PrP^C to PrP^{Sc} in cell line models, or in cell free conversion assays, a number of conclusions regarding possible modes of action can be made.

The compounds screened for binding to the three forms of PrP^C were divided into sub-categories depending on their anti-prion activity as reported in the literature. From the screening, a number of compounds, which showed binding to $huPrP^C$, t-$huPrP^C$ and $moPrP^C$ were observed. These can be classed as either multiple site binders (>130%RU_{max}), strong 1:1 binders (>50–129%RU_{max}), or weak to moderate binders in a 1:1 binding model (>5–49%RU_{max}). A compound was classed as not binding if the response was small enough to be termed not significant after visual assessment of the appropriate sensorgrams.

3.2.1. Cell line active compounds

16 out of 21 cell line active compounds were shown to bind to $huPrP^C$ at 40 μM (Table 2). A selection of these compounds was also screened at 100 μM and against t-$huPrP^C$ and $moPrP^C$. Compounds which showed high levels of binding to $huPrP^C$ at 40 μM and after which the surface could not be completely regenerated following injection, were not rescreened against the other forms of PrP^C. Binding to t-$huPrP^C$ was, in most cases, less than that observed for the $huPrP^C$ at 40 μM, whereas no significant difference was observed between $huPrP^C$ and $moPrP^C$.

The anti-prion activity of azo dyes such as Congo Red is well documented. Congo Red has been shown to have anti-prion activity in a number of different systems both in vitro [45–47] and in vivo [48]. Congo Red has also been shown to bind to PrP amyloid plaques [49] and acts by preventing the accumulation of newly formed PrP^{Sc} rather than by destabilising existing PrP^{Sc}. It has been suggested that Congo Red, and similar sulfated glycans, compete with endogenous sulfated glycans (which may be involved in the formation of PrP^{Sc}) for binding at the glycosaminoglycan (GAG) site on PrP^C and thus inhibit PrP^{Sc} formation [10]. The binding of Congo Red and other azo dyes to $huPrP^C$ has been confirmed in this investigation. Congo Red was shown to bind most strongly compared with the other dyes that are known to be active in cell lines.

Derivatives of acridine and phenothiazine have also been shown to inhibit PrP^{Sc} formation in cells chronically infected with prions. Promethazine, promazine, chlorpromazine, acepromazine, imipramine and quinacrine dihydrochloride are all known to be effective in inhibiting PrP^{Sc} formation in scrapie infected cells [40] and were all assessed for binding to $huPrP^C$, t-$huPrP^C$ and $moPrP^C$. Three out of six compounds showed varied degrees of binding to $huPrP^C$ at 40 μM, two bound to $moPrP^C$, but only quinacrine also bound to t-$huPrP^C$ at 40 μM. Quinacrine has been shown previously to bind to PrP^C by SPR [50] and NMR [22]. It interacts with three C-terminal residues (Tyr225, Tyr226 and Gln227) and the results of this investigation support the NMR study and binding of quinacrine to all three species of PrP^C was observed. At 100 μM all six compounds interacted with $huPrP^C$ and $moPrP^C$ and three bound to t-$huPrP^C$. At 40 μM acetopromazine meleate salt binds weakly to both $huPrP^C$ and $moPrP^C$, but not t-$huPrP^C$. Chlorpromazine binds only weakly to $huPrP^C$ at 40 μM. However, at a much higher concentration of 100 μM, both compounds, like quinacrine, showed binding to all three species of PrP^C suggesting that they might share the same mode of action as quinacrine. It is interesting to know that promethazine hydrochloride, promazine hydrochloride, and imipramine hydrochloride showed some binding to both $huPrP^C$ and $moPrP^C$ at 100 μM, but none to t-$huPrP^C$ showing that they are N-terminal binders and may not act by the same way as quinacrine. The mechanism of action of these compounds is not understood, but it is possible that they may interfere with the conversion of PrP^C to PrP^{Sc}. The results presented here suggest that the direct binding of phenothiazines to PrP^C may play a significant role in the mode of action. The binding of quinacrine to $huPrP^C$ is stronger than the other active phenothiazines examined in this study; it is possible that its effectiveness is related to its affinity for PrP^C as there appears to be a correlation between strength of binding and activity in cell line models. It has been suggested that concentration of phenothiazines by lysosomal trapping may lead to an increase in oxygen species, which may in turn cause PrPs to become more susceptible to protease action at lysosomal pH [51].

A collection of compounds that were shown to be active in a scrapie infected cell line by Perrier et al. were also screened for binding to $huPrP^C$. These compounds are mimics of the dominant negative PrP^C mutants that inhibit the formation of PrP^{Sc} [2] and it is believed that these compounds act to prevent prion replication by blocking PrP^C/protein X interaction, although no direct evidence for this is available. The results of this investigation show that direct binding to PrP^C may be involved in the mechanism of action of some of these compounds. However, the binding of these compounds to $huPrP^C$, t-$huPrP^C$ and $moPrP^C$ varies widely, and those compounds that are the most active in cell lines do not necessarily bind most strongly to $huPrP^C$. 2-Amino-6-[(2-aminophenyl)thio]-4-(2-furyl)-pyridine-3,5-dicarbonitile (Cp-60), which is an inhibitor of PrP^{Sc} replication in cell line assays [2], did not bind to $huPrP^C$ at either 40 or 100 μM. The analogue of Cp-60, 2-amino-6-[(4-chlorophenyl)thio]-4-(2-furyl)pyridine-3,5-dicarbonitrile (A4), which has a similar IC_{50} to Cp-60, showed clear binding to all three species of PrP^C at 40 μM. The other two analogues A3 and A5 seem to be selective towards t-$huPrP^C$. As no direct evidence is available as to the mechanism of action of these compounds, and whether the effects observed are caused by disruption of PrP^C/protein X interactions, it is possible that the binding to PrP^C observed, especially the binding to the C-terminal of PrP^C, may play a role in the mechanism of inhibition.

Generally, the binding of dicarbonitiriles to PrP^C is relatively weak when compared to compounds such as Congo Red, which may be further evidence as to their differing modes of action. Compounds that are active in cell lines, but that bind weakly to PrP^C, may elicit their effects through interaction with other factors such as protein X.

1,N^6-ethenoadenosine-5′-monophosphate disodium salt (Cp-7) did not bind to either form of PrP^C at any of the concentrations used. As this compound has been shown to produce a non-classic dose response in cell line studies it is possible that binding to

Table 2
Observed binding (%RU_{max}) of cell line active compounds screened against three forms of PrP^C at 40 μM

Compound	huPrP^C %RU_{max} ± S.D.	t-huPrP^C %RU_{max} ± S.D.	moPrP^C %RU_{max} ± S.D.	Reference, activity in cell line
Dyes[a]				
Congo Red	**1782.8**	n.a.	n.a.	[38,39,41] (IC_{50} = 1 μM) [43] (IC_{50} = 7.5 μM)
Evans blue	**809.8**	n.a.	n.a.	[39,41]
Thioflavine S	**382.3**	n.a.	n.a.	[39,40]
Primuline	**326.3**	n.a.	n.a.	[39,41]
Trypan blue	**261.3**	n.a.	n.a.	[39]
Direct Red 80 (Sirius Red)	**82.0**	n.a.	n.a.	[39]
Phenothiazine derivatives				
Quinacrine dihydrochloride	**88.4 ± 1.0**[b]	**36.9 ± 0.5**[b]	**101.7 ± 0.7**[b]	[36,37] (IC_{50} = 300–400 nM)
Chlorpromazine hydrochloride	**13.9 ± 0.9**[b]	−2.3 ± 0.2[b]	2.3 ± 0.6[b]	[37] (IC_{50} = 2 μM)
Promethazine hydrochloride	3.7 ± 0.7[b]	−3.2 ± 0.7	−2.6 ± 0.9[b]	[37] (IC_{50} = 8 μM)
Promazine hydrochloride	7.1 ± 1.7[b]	−6.7 ± 0.4	1.4 ± 2.7[b]	[37] (IC_{50} = 5 μM)
Acetopromazine maleate salt	**11.1 ± 0.4**[b]	−0.9 ± 0.2[b]	**6.0 ± 0.6**[b]	[37] (IC_{50} = 5 μM)
Imipramine hydrochloride	3.2 ± 0.2[b]	−5.3 ± 0.2	−2.8 ± 0.3[b]	[37] (IC_{50} = 10 μM)
Dicarbonitriles				
2-Amino-6-[(4-chlorophenyl) thio]-4-(2-furyl)pyridine-3,5-dicarbonitrile	**84.7 ± 5.3**[b]	**15.4 ± 1.2**[b]	**75.4 ± 4.5**[b]	[2] Cp-60 analogue A4 (IC_{50} = 18.6 μM)
2-Amino-6-[(4-chlorophenyl) thio]-4-phenyl pyridine-3,5-dicarbonitrile	0.2 ± 0.2	**11.4 ± 1.7**[b]	1.9 ± 0.5	[2] Cp-60 analogue A3 (IC_{50} = 35 μM)
2-Amino-6-[(2-aminophenyl) thio]-4-(2-furyl)pyridine-3,5-dicarbonitile	−0.3 ± 1.1	−2.1 ± 0.4	−1.2 ± 1.3	[2] Cp-60 (IC_{50} = 18 μM)
2-Amino-6-[(4-chlorophenyl) thio]-4-(2-thienyl)pyridine-3,5-dicarbonitrile	−3.8 ± 3.2	**16.7 ± 2.2**[b]	−1.1 ± 3.3	[2] Cp-60 analogue A5 (IC_{50} = 15.5 μM)
Nucleic acids				
1,N^6-ethenoadenosine 5c-monophosphate disodium salt	5.03 ± 0.9	0.8 ± 0.6	1.7 ± 1.8	[2] Cp-7 (not classic dose response)
2′,3′-Di-*O*-acetyladenosine	4.3 ± 0.2	1.1 ± 0.3	1.4 ± 0.1	[2] Cp-32 (IC_{50} = 60 μM)
Miscellaneous compounds				
Copper(II) phthalocyanine tetrasulfonic acid tetra-sodium salt[b]	**311.3**	n.a.	n.a.	Phthalocyanine [33] (PrP^{Sc} 3% of control at 10 μg/Ml)
Valproic acid[b]	**139.8**	n.a.	n.a.	Antiepileptic drug [35] (treatment of ScN2a cells causes increase in PrP^{Sc})
Suramine	**399.3 ± 0.9**[b]	**128.1 ± 2.1**[b]	**363.5 ± 1.5**[b]	Lysosomotropic agent [36] (IC_{50} = 12.3 μM)

Bold values indicate compounds classed as binding to PrP^C at 40 μM. Results are mean ± S.D. of three individual injections; n.a.: compound was not screened against the PrP^C form as indicated.
[a] SPR surface could not be completely regenerated following injection of compound, therefore results are from single injections only.
[b] Compound showed binding at 100 μM.

PrP^C does not play a role in its mechanism of action. Similar results were observed for 2,3-di-*O*-acetly adenosine.

Tetrapyrrole compounds such as porphyrins and phthalocyanines have been shown to be inhibitors of PrP^{Sc} [36]. Cell free conversion experiments suggest that the mechanism of action involves direct interactions with PrP^C or PrP^{Sc}. Copper(II) phthalocyanine tetrasulfonic acid tetrasodium salt was selected as a compound to represent this class and it clearly exhibited binding to huPrP^C by SPR. The degree of binding was similar to that of the dyes tested, which is not unexpected as tetrapyrroles bear a structural resemblance to Congo Red and are also known to bind strongly to many proteins, causing changes in protein conformation. The strong binding of copper(II) phthalocyanine tetrasulfonic acid tetrasodium salt to huPrP^C suggests that it is this direct interaction with PrP^C that may be responsible for the effects observed in cell line and cell free conversion studies. This binding to PrP^C might interfere with the interaction of PrP^C with PrP^{Sc} or other factors involved in the conversion process.

The binding of another compound, valproic acid, to huPrP^C was demonstrated. It has been shown to cause an increase in levels of PrP^C and PrP^{Sc} in scrapie infected neuroblastoma cells by several orders of magnitude [38]. Valproic acid is metabolised in vivo into a range of metabolites and it is possible that one of these metabolites is responsible for the effects observed in cell line studies, or that direct binding of valproic acid to huPrP^C, as observed in this investigation, could be directly responsible for the increase in PrP^{Sc} levels through an unknown mechanism. Metabolites of valproic acid are well known to react with nucleophiles of cellular proteins and this may also be the cause of the effects observed in cell line assays.

Suramine is a good inhibitor for PrP^{Sc} formation in cell line studies. It is a polysulfonated napthylurea, which has

Table 3
Observed binding (%RU_{max}) of cell line inactive compounds screened against three forms of PrP^C at 40 μM

Compound	huPrP^C %RU_{max} ± S.D.	t-huPrP^C %RU_{max} ± S.D.	moPrP^C %RU_{max} ± S.D.
Diverse compounds [2]			
4′,6-Diamidino-2-phenyl indole dihydrochloride (DAPI) (Cp-14)	**258.9 ± 3.1**[a]	**95.3 ± 1.7**[a]	**244.3 ± 1.5**[a]
3-Nitro-4,4′-methylene dianiline (Cp-47)	**12.6 ± 1.7**[a]	0.9 ± 1.7[a]	**11.9 ± 1.4**[a]
Urocanic acid (Cp-39)	1.4 ± 3.4	−10.9 ± 1.1	−13.3 ± 3.7
Allantoic acid (Cp-38)	1.8 ± 1.6	−7.9 ± 0.8	−9.9 ± 0.6
{[Imino(2-methoxyanilino)-methyl]amino}methanimidamide hydrochloride (Cp-59)	4.1 ± 1.0	−2.5 ± 1.0	2.5 ± 1.5
4-Amino-1-(2-furyl methyl)-2-(methyl thio)-1H-imidazole-5-carbonitrile (Cp-76)	−0.6 ± 0.7	−3.5 ± 0.7	0.7 ± 1.4
2-Amino-4,6-di(2-furyl)-4H-pyran-3,5-dicarbonitrile (Cp-75)	−5.7 ± 0.3	−4.5 ± 0.6	−5.9 ± 0.9
Amino acid/peptide derivatives [2]			
Z-phenyl arginyl-7-amido-4-methyl oumarin (Cp-73)[b]	**483.3**	n.a.	n.a.
PGlu-Gly-Arg-Phe amide acetate salt (Cp-20)[b]	**60.7**	n.a.	n.a.
Z-Arg-*O*-bzl hydrochloride (Cp-54)	**29.6 ± 0.7**[a]	**10.4 ± 0.5**[a]	**25.5 ± 0.3**[a]
MTH-DL-arginine hydrochloride (Cp-34)	2.7 ± 1.4[a]	−3.9 ± 0.3[a]	−5.1 ± 0.9[a]
H-Ala-Arg-OH acetate (Cp-5)	2.8 ± 1.1	−5.8 ± 0.6	−5.8 ± 1.0
Chloroacetyl-DL-norleucine (Cp-6)	0.8 ± 0.9	−3.7 ± 0.5	−3.6 ± 0.9
N-phthaloyl-DL-histidine (Cp-45)	2.7 ± 1.3	−5.4 ± 2.2	−4.7 ± 1.4
Water soluble antibiotics [2]			
Ribostamycin sulfate salt (Cp-21)	**7.4 ± 0.8**[a]	−0.5 ± 0.9[a]	1.1 ± 0.8[a]
Geneticin disulfate salt (Cp-19)	1.0 ± 0.5[a]	−1.6 ± 0.6[a]	−2.6 ± 0.5[a]
Sisomycin (Cp-23)	**9.4 ± 0.1**[a]	2.5 ± 0.1[a]	**6.4 ± 0.1**[a]
Streptomycin sulfate salt (Cp-21)	**3.0 ± 0.1**[a]	0.4 ± 0.1[a]	1.0 ± 0.2[a]
Butirosin disulfate (Cp-15)	3.7 ± 0.4	−2.1 ± 0.2	1.1 ± 0.5
Nucleic acids [2]			
Adenosine 2′,3′-cyclic monophosphate sodium salt (Cp-29)	4.8 ± 0.5	−4.2 ± 0.4	−2.9 ± 0.3
Adenylyl (3′,5′) cytidine (Cp-27)	5.3 ± 1.0	−1.7 ± 0.3	−0.4 ± 0.5
Guanosine 2′- and 3′-monophosphate (Cp-33)	3.6 ± 2.0	−4.2 ± 0.4	−2.8 ± 1.3
Phenothiazine derivatives [37]			
Haloperidol	**24.6 ± 2.5**[a]	3.6 ± 0.8[a]	**20.2 ± 3.1**[a]
2-Chloro-phenothiazine	4.6 ± 0.2[a]	−6.8 ± 0.8[a]	−4.6 ± 0.6[a]
Clozapine	**8.9 ± 2.3**[a]	−1.1 ± 0.5[a]	3.3 ± 2.0[a]
Carbamazepine	−0.5 ± 1.0	−9.2 ± 0.4	−4.8 ± 2.3

Bold values indicate compounds classed as binding to PrP^C at 40 μM. Results are mean ± S.D. of three individual injections; n.a.: compound was not screened against the form of PrP^C as indicated.

[a] Compound showed binding when screened at 100 μM.

[b] SPR surface could not be completely regenerated following injection of compound, therefore results are from single injections only.

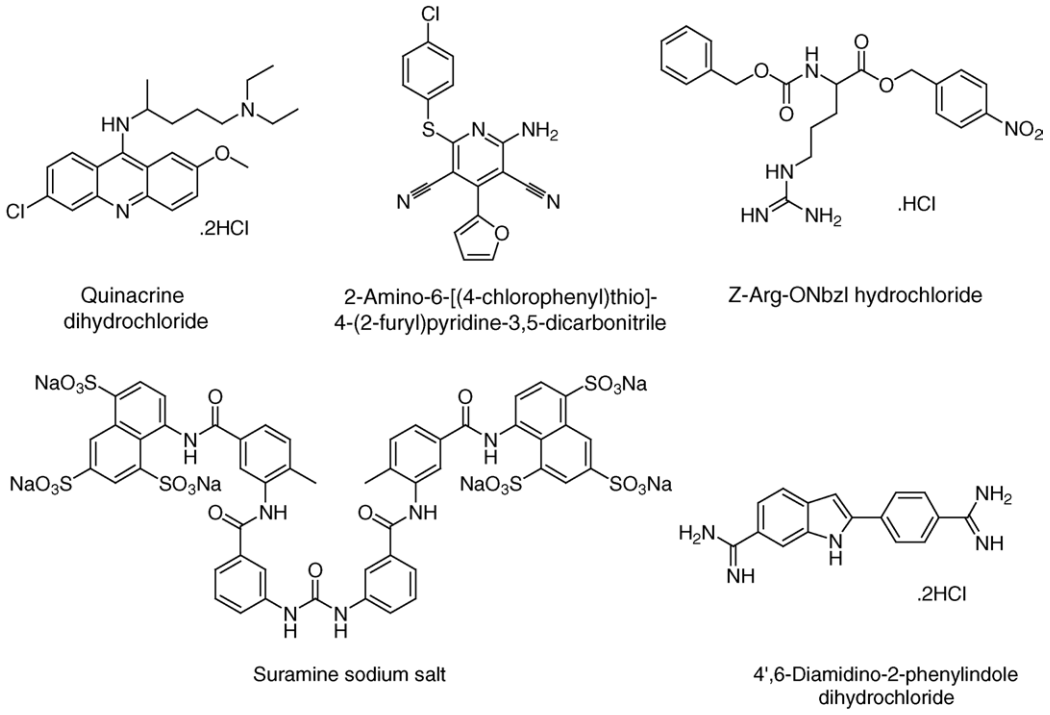
been associated with a large number of biological activities. Like quinacrine, it is also a lysosomotropic compound (substances that are taken up selectively into lysosomes) and has been suggested to prevent PrP^{Sc} formation indirectly, or by reducing the metabolic half-life of PrP^{Sc} [39]. The endocytic pathway is believed to be involved in the conversion process from PrP^C to PrP^{Sc} and suramine may act via a similar mechanism to Congo Red and PPS in this respect.

3.2.2. Cell line inactive compounds

A group of 26 compounds were screened for binding to huPrP^C, t-huPrP^C and moPrP^C (Table 3), which comprised of compounds which had previously been screened for activity in prion infected cell lines from a study by Perrier et al. [2], and those from a study by Korth et al. [40]. Ten of the 26 compounds screened showed varied levels of binding to huPrP^C at 40 μM. The binding of a further three compounds to huPrP^C was only observed at 100 μM.

Eight of the 22 compounds assessed from a study by Perrier et al. [2] were shown to bind to huPrP^C with varying strengths at 40 or 100 μM. This is not unexpected because these compounds were selected as mimics of the dominant negative PrP^C mutants and were all chosen using the same pharmacophore as the active compounds, many of which showed binding to huPrP^C. Twenty of these compounds were also assessed for binding to t-huPrP^C and moPrP^C, of which eight bound weakly to both proteins at either 40 or 100 μM. One of the diverse compounds, DAPI (Cp-14), which showed strong binding to all forms of PrP^C at 40 and 100 μM, is used for staining DNA in fluorescence microscopy and binds to cellular proteins such as tubulin, and therefore its strong affinity for PrP^C was not unexpected.

Cp-73 and Cp-20 bound to huPrP^C, but the surface could not be regenerated after the binding and were therefore excluded from further study. The remaining diverse compounds bound very weakly or did not bind to PrP^C. Three of the seven amino acid/peptide derivatives bound to huPrP^C to varying degrees. The results suggest that some small peptides bind to PrP^C,



Compound	huPrP^C K_D (μM)	moPrP^C K_D (μM)
Quinacrine dihydrochloride	**15**	**-**
2-Amino-6-[(4-chlorophenyl) thio]-4-(2-furyl)pyridine-3,5-dicarbonitrile	**n/a***	**n/a***
Suramine	**8.41**	**7.31**
4',6-Diamidino-2-phenyl indole dihydrochloride (DAPI) (Cp-14)	**1.13**	**1.12**
Z-Arg-ONbzl hydrochloride (Cp-54)	**103**	**-**

* Unable to get a good fit. Estimated K_D = 20 μM (huPrP^C); 55μM (moPrP^C).

Fig. 2. Compounds for which dissociation constants (K_D) were determined.

although they are likely to be metabolised within cells and this binding may be lost. Four out of five water soluble antibiotics bound to huPrP^C very weakly with varying affinities. This was not unexpected as antibiotics are known to bind reversibly to serum albumin and other tissue proteins and therefore binding to PrP^C is probably as a result of their non-specific nature. None of the three nucleic acids tested bound to any form of PrP^C, at either 40 or 100 μM concentration. In conjunction with the weak binding of the same class of compounds discussed earlier, this class of compounds is unlikely to yield potential therapeutic agents which act through the binding to PrP^{C}s.

Phenothiazine derivatives, which were inactive in cell line studies, bound to huPrP^C and moPrP^C at either 40 or 100 μM with similar strength to the cell line active phenothiazines. This demonstrates that the structural similarity of compounds may be significant for binding to huPrP^C but that this is not necessarily an indication that a compound will be active in cell line studies.

3.3. Kinetic studies of the selected binders

The representative best binders in each substructural class were subjected to kinetic studies (Fig. 2). Quinacrine binding to huPrP^C was examined at concentrations ranging from 0 to 30 μM (Fig. 3A and B). Nonlinear regression of quinacrine binding (RU) as a function of concentration gave a dissociation constant, K_D, of 15 μM. The IC_{50} for quinacrine in cell lines is much lower than the K_D calculated using SPR, and other in vitro studies (K_D from NMR is ∼4.6 μM). This low IC_{50} value in cell lines may be due to the intracellular concentration of quinacrine by cell membranes [52]. Similarly other members of the phenothiazine family might also be expected to have a higher K_D, than IC_{50} in cell lines. Therefore, an intracellular concentration effect may be occurring with all members of this family.

The binding kinetics for polysulfonated suramine to huPrP^C and moPrP^C are similar and in agreement with its IC_{50} in cell line studies. It is interesting to see that DAPI gave the lowest K_D value, but is inactive in the cell line studies. It may be due to

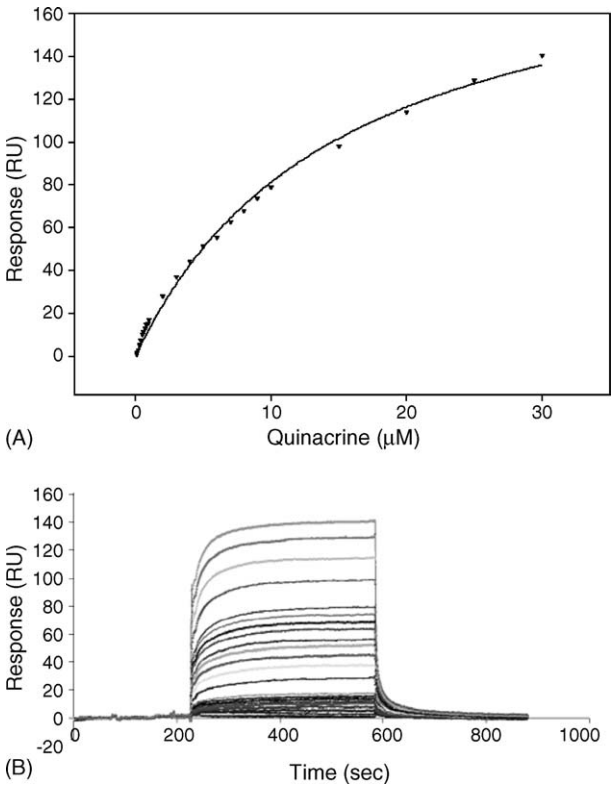


Fig. 3. Binding of quinacrine to huPrP^C immobilized on a sensor chip. (A) Nonlinear regression of the binding data. The value for the dissociation constant (K_D) of the binding system is 15 μM. (B) Sensorgrams of quinacrine binding to immobilized huPrP^C. The concentrations of quinacrine used ranged from 0 to 30 μM.

the fact that the labile amidino group can easily be metabloised in the cell or due to its polarity, makes it unable to cross the plasma membrane. *Z*-Arg-*O*-bnzl hydrochloride (Cp-54) also has an amidino group whilst the K_D is much higher and it is also inactive. This makes the first speculation more convincing. Further kinetic studies are required for Cp-60 analogue A4 to obtain a reliable K_D or confirm it is a non-specific binder.

In summary, screening results from a library of 47 compounds of known activity in cell line and cell free conversion studies by SPR showed some interesting trends:

- In general, compounds that bind to both huPrP^C and moPrP^C showed slightly stronger binding towards huPrP^C except quinacrine which binds to moPrP^C preferably. For compounds (suramine, Cp-14, Cp-54, etc.) which bind to huPrP^C and/or moPrP^C, but bind more weakly to t-huPrP^C, it is most likely that they bind preferably towards the N-terminal of the protein.
- All six dyes which have been shown to be active in cell line studies bound to PrP^C very strongly. In most cases binding exceeded 100%RU$_{max}$ and the bound compounds could not be removed from the chip surface, indicating that these are mostly non-specific. However, it is quite possible that their cell line activity may be due to binding to PrP^C.
- Nucleic acid-based compounds are unlikely to be a useful lead series for therapeutics targeting PrP^C because although two out five showed some inconsistent cell line activity, none of the five compounds showed any binding to any of the three species of PrP^C.
- Six out of 10 compounds in the phenothiazine family showed cell line activity and they all show binding towards PrP^C to some degree. This indicates that their mode of action might be through the binding to PrP^C as quinacrine was also observed to bind to this protein.
- Four out of six dicarbonitrile-based compounds showed cell line activity, but their binding to PrP^C varied, therefore it can only be speculation that their mode of action is via PrP^C binding, to disrupt the interaction between PrP^C and protein X as was reported. However, analogues A4, A3 and A5 all showed some selective binding towards t-huPrP^C. This class of compounds might be selected as a lead structure for developing specific binders for the C-terminal of huPrP^C.

4. Conclusions

An SPR system using Biacore 3000 has been identified as a useful tool for the screening of compounds binding to PrP^C. An optimal immobilization procedure was developed, which significantly reduced the observed baseline increase. The intensive studies of interactions between PrP^C and CM-dextran revealed that the interactions between immobilized PrP^C and the CM-dextran matrix are fast and irreversible. It results in the heparin binding site on the N-terminal of PrP^C becoming unavailable therefore heparin binding is weakened and lost over time. It also causes the slight conformational change and repositioning of PrP^C on the chip surface leading to an increased exposure of the C-terminal of PrP^C, therefore the quinacrine binding increases over the time.

The optimized immobilization and screening protocol enabled reproducible binding measurements to be made and led to the development of a more robust assay, which was used to screen a library of potential prion disease therapeutics against huPrP^C. Overall, the screening results show that direct binding to PrP^C is likely to play a significant role in the anti-prion activity of a number of compounds. While binding to PrP^C itself does not directly indicate that a compound will have an anti-prion effect in cell line assays, a compound which binds to PrP^C is more likely to have an effect than once which does not. The exact role this binding plays in the mechanism of action of these compounds is not fully understood. However, stabilisation of the native structure with a ligand through binding to specific sites, which makes conversion to PrP^{Sc} less favourable, or by disrupting the interaction of PrP^C with PrP^{Sc}, or other cofactors such as protein X, may be possible mechanisms of action. Further investigation of active and inactive compounds that bind to PrP^C may help to provide an insight into the mechanism of action of potential prion therapeutics.

The SPR ligand/prion protein binding assay described is currently employed in the screening of combinatorial libraries as part of a medicinal chemistry program towards identification of novel prion therapeutics. Further results will be reported elsewhere.

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