

## Surface Plasmon Resonance Analysis for the Screening of Anti-prion Compounds

Satoshi KAWATAKE,<sup>a</sup> Yuki NISHIMURA,<sup>a</sup> Suehiro SAKAGUCHI,<sup>b</sup> Toru IWAKI,<sup>c</sup> and Katsumi DOH-URA<sup>\*a</sup>

<sup>a</sup> Department of Prion Research, Tohoku University; Sendai 980–8575, Japan; <sup>b</sup> Department of Molecular Microbiology and Immunology, Nagasaki University; Nagasaki 852–8523, Japan; and <sup>c</sup> Department of Neuropathology, Neurological Institute, Kyushu University; Fukuoka 812–8582, Japan.

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**The interaction of anti-prion compounds and amyloid binding dyes with a carboxy-terminal domain of prion protein (PrP121–231) was examined using surface plasmon resonance (SPR) and compared with inhibition activities of abnormal PrP formation in scrapie-infected cells. Most examined compounds had affinities for PrP121–231: antimalarials had low affinities, whereas Congo red, phthalocyanine and thioflavin S had high affinities. The SPR binding response correlated with the inhibition activity of abnormal PrP formation. Several drugs were screened using SPR to verify the findings: propranolol was identified as a new anti-prion compound. This fact indicates that drug screenings by this assay are useful.**

**Key words** anti-prion compound; surface plasmon resonance; scrapie-infected cell; screening; recombinant prion protein

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheinker syndrome in humans, and scrapie, bovine spongiform encephalopathy and chronic wasting disease in animals. These disorders are characterized by accumulation in the brain of an abnormal isoform of prion protein (PrP), which includes a high beta-sheet content and is resistant to digestion with proteinase K.<sup>1)</sup> Recent outbreaks of variant Creutzfeldt–Jakob disease<sup>2)</sup> and iatrogenic Creutzfeldt–Jakob disease through use of cadaveric growth hormone or dura grafts<sup>3)</sup> in younger people have necessitated the development of suitable therapies. Compounds such as antimalarials and amyloid binding dyes are known to possess anti-prion activity *in vitro* or *in vivo*.<sup>4–14)</sup> Among them, Congo red and quinacrine are known to bind directly to PrP and thereby strongly inhibit proteinase K-resistant PrP (PrPres) formation.<sup>15,16)</sup> However, it remains unclear whether or not other anti-prion compounds and amyloid binding dyes interact directly with PrP. This study analyzed interactions of some previously reported anti-prion compounds<sup>4,7,11,17,18)</sup> and popularly used amyloid binding dyes with recombinant PrP using surface plasmon resonance (SPR). In addition, we evaluated whether SPR assay is useful as a screening tool for anti-prion compounds.

### MATERIALS AND METHODS

**Compounds** Compounds used in the study (Fig. 1) were obtained from Sigma Aldrich Corp. (quinacrine dihydrochloride (QC, MW: 400.0), quinine hydrochloride (QN, MW: 324.4), thioflavin T (ThT, MW: 283.4, dye content 65%), thioflavin S (ThS, MW: undetermined), propranolol (MW: 295.8), promethazine hydrochloride (MW: 284.4), carbamazepine (MW: 236.3) and theophylline (MW: 180.2)), Aldrich (chloroquine diphosphate (CQ, MW: 319.9), and Congo red (CR, MW: 696.7, dye content 97%)), ICN (phthalocyanine tetrasulfonate (PcTS, MW: 922.7)), Wako Pure Chemical Industries Ltd. (Tokyo, Japan) (tetracycline hydrochloride (TC, MW: 444.4), diazepam (MW: 284.7), folic

acid (MW: 441.4) and phenytoin (MW: 252.3)) or Nacalai Tesque (Tokyo, Japan) (testosterone (MW: 288.4)). All compounds were prepared as 20 mM stock solutions in water or dimethyl sulfoxide.

**SPR Analysis** The SPR analysis was performed using an

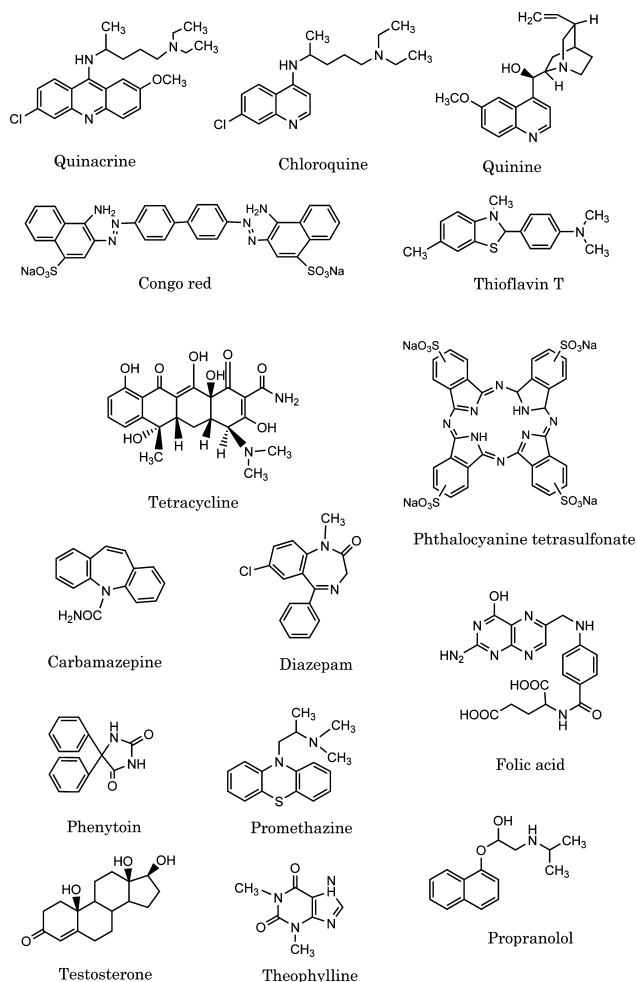


Fig. 1. Structures of Compounds or Drugs Used in the Study

\* To whom correspondence should be addressed. e-mail: doh-ura@mail.tains.tohoku.ac.jp

optical biosensor (Biacore AB, Uppsala, Sweden) equipped with a CM5 sensor chip. Recombinant mouse PrP was prepared as described previously<sup>19,20</sup> and immobilized on a biosensor chip at a density of *ca.* 3000 resonance units (RU) using amine coupling.<sup>21</sup> Test compounds were diluted to 100  $\mu\text{M}$  with running buffer (70 mM NaCl, 53 mM  $\text{Na}_2\text{HPO}_4$ , 12.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4) and contained 0.5% DMSO. After they were confirmed to be in solution without precipitation or aggregation, they were injected over the PrP flow cell and the reference for either 60 s at a flow rate of 20  $\mu\text{l}/\text{min}$  (low-affinity compounds) or 90 s at a flow rate of 30  $\mu\text{l}/\text{min}$  (high-affinity compounds). The dissociation phase was monitored for 60 s (low-affinity compounds) or 270 s (high-affinity compounds). The flow cell was washed with 10 mM NaOH or 0.01% Triton X-100 for 30 s between each sample injection. Buffer blanks for double referencing were injected before sample analyses.<sup>22</sup>

The full-length recombinant of mouse PrP (residues 23–231) was used initially in the experiment, but it was easily degraded during SPR analysis in the amino-terminal portions attributable to an unidentified mechanism. For that reason, the carboxy-terminal polypeptide (residues 121–231; PrP121–231), which represents the only autonomous folding unit of PrP with a defined three-dimensional structure,<sup>19,23,24</sup> was used in this study.

Every PrP-immobilized biosensor chip used in the study was confirmed to respond almost the same and was standardized by the measurement of QC before its use for sample analyses.

**Data Analysis** The binding response, which is an index for estimating the interaction of a compound with molecules sited on a biosensor chip, is obtained from the equilibrium response ( $R_{\text{eq}}$ ) value or the maximum response value in the sensorgram divided by the molecular weight.<sup>25</sup> In this study, the binding response of a compound was standardized by calibrating with QC, whose binding response was designated as 100 RU/Da. For low-affinity compounds, the dissociation constant ( $K_D$ ) based on the  $R_{\text{eq}}$  state was calculated from data at doses ranging from 10  $\mu\text{M}$  to 1 mM by either steady-state analysis using BIAevaluation software (ver. 3.0; Biacore AB) or Scatchard plot analysis. On the other hand, the  $K_D$  for high-affinity compound CR or PcTS was deduced after the data were fit to a binding model assuming a bivalent analyte in BIAevaluation software. The fitting was performed in such a way that the  $\chi^2$  value representing the statistical closeness of curve-fitting became the lowest. It was recommended ideally to be below 10.

Statistical linear correlation was evaluated using Pearson's correlation coefficient; Fisher's *r* to *z* method was used to calculate the *p* values. Simple linear regression analysis was also performed.

**Anti-prion Activity Assay** Anti-prion activity of a compound was assayed by measuring its 50% inhibition doses ( $\text{IC}_{50}$ ) for PrPres formation in scrapie-infected neuroblastoma (ScNB) cells as described in previous reports.<sup>7,11,12</sup> Briefly, compounds were added at designated concentrations to the medium when cells were passed at 10% confluency. Cells were allowed to grow to confluence and lysed with lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS). Lysates were digested with 10  $\mu\text{g}/\text{ml}$  proteinase K for 30 min and centrifuged at 100000 $\times g$  for 30 min at 4  $^\circ\text{C}$ . The

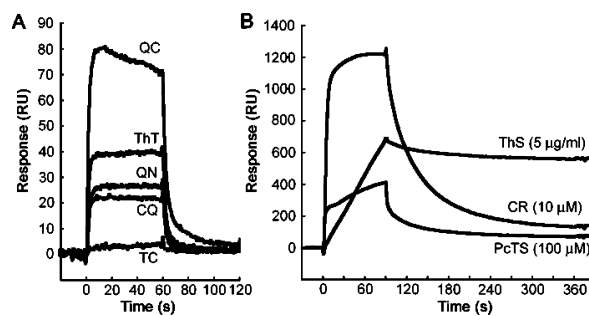


Fig. 2. Interactions of Anti-prion Compounds with PrP121–231

(A) Sensorgrams of the low-affinity compounds quinacrine (QC), chloroquine (CQ), quinine (QN), thioflavin T (ThT) and tetracycline (TC), all at 100  $\mu\text{M}$ . (B) Sensorgrams of the high-affinity compounds Congo red (CR, 10  $\mu\text{M}$ ), phthalocyanine tetrasulfonate (PcTS, 100  $\mu\text{M}$ ) and thioflavin S (ThS, 5  $\mu\text{g}/\text{ml}$ ).

pellets were resuspended in sample loading buffer and boiled. Samples were separated using electrophoresis on a 15% Tris-glycine-SDS-polyacrylamide gel and electroblotted. PrPres was detected using an antibody SAF83 (1 : 5000; SPI-Bio, France), followed by an alkaline phosphatase-conjugated secondary antibody. Immunoreactive signals were visualized using CDP-Star detection reagent (Amersham Biosciences Corp., U.S.A.) and were analyzed densitometrically. Three independent assays were performed in each experiment.

## RESULTS

**Interaction of Anti-prion Compounds with PrP** The SPR sensorgrams of ThT and antimalarials such as QC, QN and CQ (each at 100  $\mu\text{M}$ ) demonstrated weak signal responses of less than 100 RU (Fig. 2A). The responses of these compounds reached equilibrium ( $R_{\text{eq}}$ ) within a few seconds and returned to the baseline very rapidly after dissociation. These sensorgrams were typical for low-affinity interactions: TC showed almost no response. On the other hand, all sensorgrams of high-affinity compounds, such as CR, PcTS and ThS, showed much stronger responses and individual characteristic curves that differed from those of the low-affinity compounds (Fig. 2B). The CR (10  $\mu\text{M}$ ) showed the strongest signal, which was greater than 1200 RU: this decreased very slowly in the dissociation phase. The signal responses for PcTS (100  $\mu\text{M}$ ) and ThS (5  $\mu\text{g}/\text{ml}$ ) showed that neither reached the  $R_{\text{eq}}$  state within the association phase or returned to the baseline within the dissociation phase. In particular, ThS was only slightly dissociated and remained bound. This sensorgram resembled the sensorgram of biquinoline, an effective inhibitor of PrPres formation in ScNB cells ( $\text{IC}_{50}=3 \text{ nM}$ ).<sup>11</sup>

**$K_D$  Determination** The dose response curve for QC appeared to be monophasic and to reach a saturation level at higher concentrations; its dissociation constant ( $K_D$ ) value was calculated as 1.1 mM or 0.9 mM using steady-state analysis or Scatchard plot analysis, respectively (Figs. 3A–C). Vogtherr *et al.*<sup>16</sup> reported the dissociation constant ( $K_D=4.6 \text{ mM}$ ) of the complex of QC and human PrP 121–230 analyzed by nuclear magnetic resonance (NMR) spectroscopy. This value was almost comparable to the  $K_D$  value obtained in this study, indicating that the method used in this study was relevant. The other two low-affinity compounds, QN and

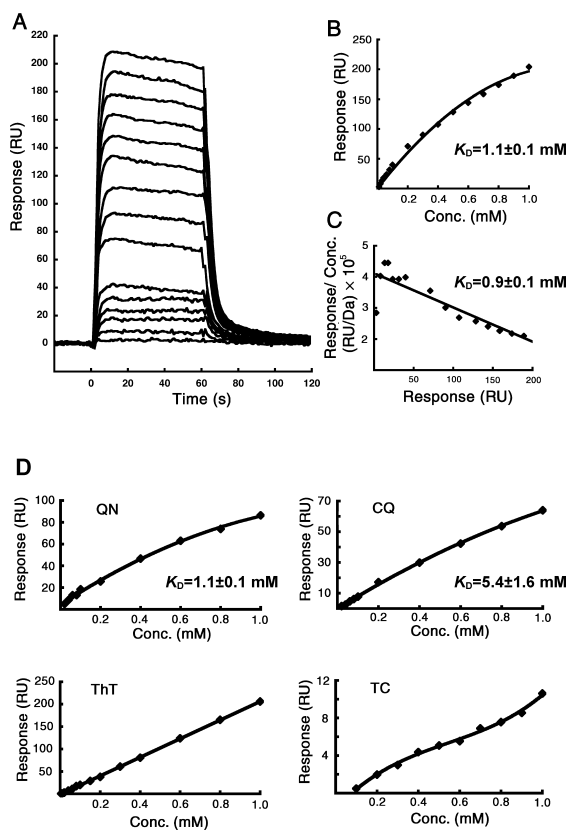


Fig. 3. Kinetic Analyses of Low Affinity Compounds

(A) Sensorgrams, (B) dose response curve and (C) scatchard plot of QC. (D) Dose response curves of QN, CQ, ThT, and TC.

CQ, respectively showed a similar monophasic pattern in dose response curves, yielding  $K_D$  of 1.1 mM and 5.4 mM (Fig. 3D). These  $K_D$  values, however, were of rough estimation and might be a little underscored due to lack of the data at concentrations of more than 1 mM. Unstable solubility of the compounds at such high concentrations hindered further analyses.

On the other hand, ThT gave a linear dose–response curve within a concentration of up to 1 mM and TC showed a biphasic pattern (Fig. 3D). Therefore, the saturation levels and  $K_D$  values of these compounds could not be determined, indicating that these compounds have a very low or no affinity with PrP121–231. Of them, TC is known to revert abnormal physicochemical properties of PrPres *in vitro*,<sup>18)</sup> and interaction between TC and human PrP 106–126 peptides is revealed by NMR analysis.<sup>26)</sup> Their data appear to be inconsistent with the data in this study. However, this discrepancy might be attributable to the lack of a TC binding site in the PrP121–231 used in our study.

Each sensorgram of high affinity compounds showed a very slow dissociation phase and was individually characteristic (Fig. 4). The structural and stoichiometric binding details of the compounds with PrP121–231 have not yet been established, but CR or PcTS is a symmetrical molecule and either half of the molecule has anti-prion activity (Doh-ura K, unpublished data). Consequently, the  $K_D$  value for the compound was deduced after the data were fit to a binding model assuming a bivalent analyte. The  $K_D$  of CR was calculated to be 1.6  $\mu$ M from the sensorgrams of 1, 2, 3.3 and 5  $\mu$ M ( $\chi^2=20.9\pm 2.1$ ) (Fig. 4A). The  $K_D$  of PcTS was calculated as

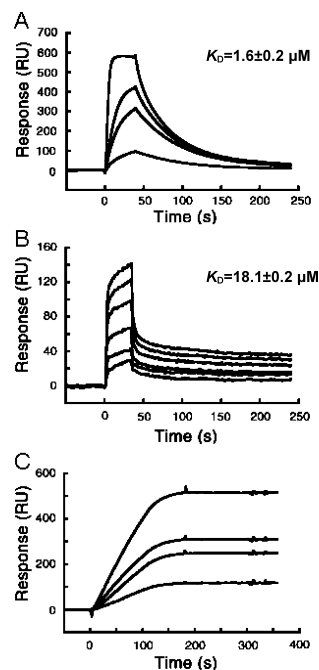


Fig. 4. Kinetic Analyses of High Affinity Compounds

(A) Sensorgrams of CR at concentrations of 1, 2, 3.3 and 5  $\mu$ M, and its  $K_D$  value. (B) Sensorgrams of PcTS at concentrations of 1, 5, 10, 50, 75 and 100  $\mu$ M, and its  $K_D$  value. (C) Sensorgrams of ThS at concentrations of 1, 2, 3 and 5  $\mu$ g/ml, and its  $K_D$  value could not be calculated because of its undetermined structure and molecular weight.

18.1  $\mu$ M from the sensorgrams of 1, 5, 10, 50, 75 and 100  $\mu$ M ( $\chi^2=28.1\pm 2.9$ ) (Fig. 4B). The  $K_D$  of ThS was incalculable to an exact degree because it is presumed to be a mixture of compounds formed by methylation and sulfonation of primulin; their structures and molecular weights have not been determined.

**Comparison between PrP Affinity and Anti-prion Activity** The  $IC_{50}$  value for the inhibition of PrPres formation in ScNB cells, either previously reported or examined in this study, was used as an anti-prion activity in this study. It was compared with the  $K_D$  or with the binding response. The latter, an index for estimating the interaction, was obtained from the  $R_{eq}$  value or the maximum response value at a concentration of 1 mM divided by the molecular weight (Table 1).

From data of all compounds except ThT, TC and ThS, statistical analyses demonstrated a significant linear correlation between the reciprocal of binding response and the  $IC_{50}$  ( $r=0.985$ ,  $p=0.0005$ ) (Fig. 5). This relation appeared to be also observed in TC, but not in ThT showing the next highest binding response to QC but no inhibition of PrPres formation within a non-toxic dose range. However, ThT demonstrated cell-toxicity at such a low dose as 0.05  $\mu$ M.

For ThS, assuming that its minimum molecular weight deduced from presumable structures was 520 Da, its binding response was estimated to be 5.03 RU/Da; the  $IC_{50}$  was estimated to be about 2  $\mu$ M, corresponding to about 1  $\mu$ g/ml. However, these values seem to be underestimates because some constituents of ThS might interact with PrP121–231 or have inhibitory activity for PrPres formation. Therefore, active constituents of ThS might be expected to inhibit PrPres formation in ScNB cells at a submicromolar dose, similar to the other high-affinity compounds.

**Screening by SPR** Findings suggested that a compound

Table 1. Binding Response, Dissociation Constant ( $K_D$ ) and 50% PrPres Inhibition Dose in ScNB Cells ( $IC_{50}$ )

Compound	Binding response <sup>a)</sup> (RU/Da)	$K_D$ <sup>b)</sup> (mM)	$IC_{50}$ <sup>c)</sup> ( $\mu$ M)
<b>Low-affinity</b>			
Quinacrine (QC)	0.25 $\pm$ 0.00	1.1 $\pm$ 0.1 (0.9 $\pm$ 0.1)	0.3 (7)
Quinine (QN)	0.05 $\pm$ 0.00	1.1 $\pm$ 0.1 (1.4 $\pm$ 0.1)	6.0 (11)
Chloroquine (CQ)	0.07 $\pm$ 0.01	5.4 $\pm$ 1.6 (3.5 $\pm$ 0.8)	4.0 (7)
Thioflavin T (ThT)	0.16 $\pm$ 0.01	n.d. <sup>d)</sup> (n.d. <sup>d)</sup> )	No effect <sup>f)</sup>
Tetracycline (TC)	0.01 $\pm$ 0.00	n.d. <sup>d)</sup> (n.d. <sup>d)</sup> )	No effect <sup>g)</sup>
<b>High-affinity</b>			
Congo red (CR)	8.74 $\pm$ 0.64	1.6 $\pm$ 0.2 $\times$ 10 <sup>-3</sup>	1.5 $\times$ 10 <sup>-2</sup> (4)
Phthalocyanine tetrasulfonate (PcTS)	1.82 $\pm$ 0.06	18.1 $\pm$ 0.2 $\times$ 10 <sup>-3</sup>	0.5 (17)
Thioflavin S (ThS)	n.d. <sup>e)</sup>	n.d. <sup>e)</sup>	ca. 1 $\mu$ g/ml

a) Binding response value was calculated from the  $R_{eq}$  value divided by the molecular weight for QC, QN, CQ and CR, or from the response value at a concentration of 1 mM divided by the molecular weight for ThT, TC and PcTS. b)  $K_D$  values were determined by steady state analysis for the low-affinity compounds or by bivalent analyte model analysis for CR and PcTS.  $K_D$  values from Scatchard plot analyses are shown in parentheses. c)  $IC_{50}$  values reported in the literature (reference shown in parentheses) or examined in this study. d) n.d.: not determined because a saturation level could not be estimated. e) n.d.: not determined because its structure and molecular weight were undetermined. f) Inhibition of PrPres formation was not observed up to a minimal toxic dose of 0.05  $\mu$ M. g) Inhibition of PrPres formation was not observed up to a minimal toxic dose of 5.0  $\mu$ M.

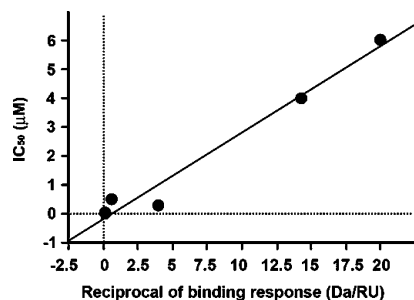


Fig. 5. Correlation between the Reciprocal of Binding Response and the  $IC_{50}$

The data were from five compounds in which both binding response and  $IC_{50}$  were determined. Correlation showed a slope of 0.298, an intercept of  $-0.156$  and a correlation coefficient of 0.971 ( $p=0.002$ ) by simple linear regression analysis.

capable of interacting with PrP121–231 might have a potency of inhibiting PrPres formation in ScNB cells. To verify this inference, several drugs were examined for either their binding response using the SPR method or their  $IC_{50}$  in ScNB cells. Eight clinically utilized drugs—carbamazepine, diazepam, folic acid, phenytoin, promethazine, propranolol, testosterone, and theophylline—all of which are low molecular weight compounds capable of crossing the blood brain barrier and share a partial structure similarity with the anti-prion compounds already reported, were examined and compared with the four anti-prion compounds (QC, QN, CQ, and ThT) (Fig. 6A).

Diazepam, promethazine and propranolol showed a higher binding response value than QN, which was the lowest binding response compound among the effective anti-prion compounds examined in this study. Among these, promethazine or propranolol inhibited PrPres formation in ScNB cells (propranolol:  $IC_{50}=0.7 \mu$ M; promethazine:  $IC_{50}<5.0 \mu$ M). Promethazine has already been reported to have anti-prion activity in ScNB cells,<sup>8)</sup> whereas propranolol is a novel compound that inhibits PrPres formation in ScNB cells. Diazepam apparently did not inhibit PrPres formation within a non-toxic dose range up to 25  $\mu$ M (Fig. 6B). Inhibitory activi-

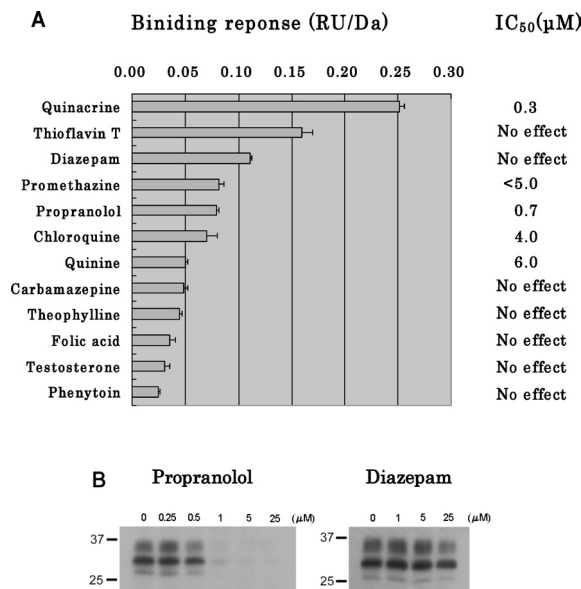


Fig. 6. Screening of Anti-prion Candidates Using the SPR Assay

(A) Binding response of each sample at 100  $\mu$ M, and its  $IC_{50}$  of PrPres formation inhibition in ScNB cells within a non-toxic dose range. (B) Inhibition analyses of PrPres formation in ScNB cells grown in the presence with propranolol or diazepam. Molecular sizes in kDa are shown at the left of each panel.

ties against PrPres formation in ScNB cells were not observed for other drugs that had lower binding response values than QN.

## DISCUSSION

We demonstrated that most anti-prion compounds examined in this study interacted with PrP121–231. The binding response of the compounds correlated with the  $IC_{50}$  of PrPres formation inhibition in ScNB cells. In addition, based on this finding, we proved that this interaction analysis using the SPR method was useful for screening to identify new candidates of anti-prion compounds. Three different *in vitro*

screening assays have been reported recently. One is yeast based,<sup>27)</sup> one uses ScN2a cells,<sup>10)</sup> and the other is based on fluorescence correlation spectroscopy.<sup>28)</sup> These assays are suitable for high-throughput screening of large compound libraries to identify novel lead molecules. The SPR method reported here, which easily assayed interactions between compounds and PrP molecules within less than 3 min per compound, is applicable to high-throughput *in vitro* assay for screening of large compound libraries if more highly performing SPR machines are used. The usefulness of this method in screening for PrP binding ligands is also reported very recently by other researchers.<sup>29)</sup>

Two chemicals, ThT and diazepam, showed high binding response but did not inhibit PrP formation within a non-toxic dose range. Of them, ThT exhibited very low or no affinity with PrP121–231 but the next highest binding response to QC. This suggests that ThT might interact with PrP121–231 non-specifically. For diazepam, similar non-specific interaction with PrP121–231 might be occurred, or the interaction might be specific but unrelated to conversion to PrPres. These inferences, however, remain unsupported by other experimental results obtained here.

On the other hand, such high-affinity compounds as CR and PcTS showed large amounts of binding to PrP121–231. One possible interpretation for this is that the compounds might have two or more binding sites per molecule. In fact, structure–activity relationship analysis for these symmetrical compounds indicates that either half of the molecule has anti-prion activity (Doh-ura K, unpublished data), and their sensorgrams looked very similar to those of anti-PrP antibodies (data not shown). The other is that the compounds might self-assemble to interact with the PrP molecule. It has long been known that CR and many other bis-azo dyes self-assemble in water solutions, and this property is proposed to associate with binding capability.<sup>30)</sup>

Instead of the full length of mouse PrP, a carboxy-terminal domain of mouse PrP (PrP121–231) was used in the study because of instability of the full length PrP during the experiment. This carboxy-terminal domain is the only autonomous folding unit of PrP with a defined three-dimensional structure<sup>19,23,24)</sup> and contains epitopes recognized by a majority of antibodies bearing anti-prion activity.<sup>31–37)</sup> Taken together with our findings suggesting that most of anti-prion compounds might exert their effects by interacting with this domain, targeting the carboxy-terminal domain should not necessarily be either inefficient or inappropriate for looking for new anti-prion compounds.

In conclusion, our study indicated that most anti-prion compounds tested here interacted with and had an affinity for recombinant PrP121–231. The SPR binding response to the PrP121–231 correlated with the anti-prion activity in ScNB cells. These observations will allow further discovery of new classes of anti-prion compounds using the SPR assay.

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